MHC CLASS II-RESTRICTED T CELL RESPONSES TO FLAVIVIRUSES

by

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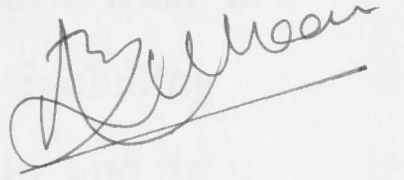
CONTENTS

STATEMENT

The experiments described in this thesis represent my own work except for the following. The splenic dendritic cells described in Chapter 3 were prepared by Ms. Cathy Woodhams. The recombinant vaccinia viruses encoding Kunjin proteins described in Chapter 4 were constructed by Dr.C.Parrish.



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ABSTRACT

The work described in this thesis was undertaken to investigate murine MHC class II-restricted presentation of flavivirus antigens, the relative efficacy of various classes of antigen-presenting cells in presenting WNV antigens to T cells and the influence of Ir genes and high APC numbers on T cell proliferation.

Initially, combined *in vivo-in vitro* protocols for the generation of WNV-specific murine T cell proliferative responses were investigated. Variables examined included the conditions necessary for priming mice, such as route and dose of virus inoculation, determination of optimum post-priming intervals for obtaining maximum proliferative responses and several *in vitro* assay conditions. A reductionist approach was employed for each variable to delineate the parameters that obscured the evaluation of antigen-specific proliferative responses. Strong proliferative responses were obtained using splenocytes enriched for responder $CD4^+$ T cells, Ia^+ stimulator populations from primed mice and antigen in the form of a 12 h lysate prepared from WNV-infected Vero cells. In order to reduce the background proliferation occurring mostly due to media containing FCS, a modified T cell proliferation assay was developed. In this assay media containing NMS were used for culturing APC and T cells in the presence of WNV antigen for 3 days, followed by a further 2 days culture in media containing EL-4 supernatant. This modification of conventional 3 day assay led to a substantial reduction in background proliferation.

The relative efficacy of macrophages, B cells and SDC in presenting WNV antigens to WNV-immune CD4+ T cells was evaluated. The results indicate that on a cell population basis *Listeria*-induced macrophages were the most efficient inducers of WNV-specific proliferative responses when compared with B cells obtained from naive or 14 day WNV-primed mice and SDC. With regard to B cells, those obtained from WNV-primed mice evoked higher responses than naive or mitogen-activated B cells. Interestingly, B cells obtained from WNV-hyper-immune mice elicited optimal responses with lower doses of antigen than naive or 14-day WNV-primed B cells. Addition of WNV-specific antibodies increased the efficiency of antigen presentation by macrophages whereas, in the case of hyperimmune B cells, these antibodies inhibited their efficiency.

The influence of Ir genes and background genes on WNV- and Kunjin-specific T cell responses was determined. Kunjin-immune T cells from the H-2^b and H-2^d MHC haplotypes predominantly recognized structural (C, prM/M and E) and membrane-associated non-structural proteins (NS1) encoded by VKV 1031 and showed weaker responses to cytosolic non-structural protein, NS5 (VKV 1022), whereas responders of the H-2^k haplotype strongly recognized the antigens encoded by VKV 1022 and VKV 1031. B cells from all the haplotypes tested, although they presented 1031 and 1022, differed from macrophages in terms of magnitude of T cell responses induced. Macrophages from BALB/b (H-2^b), BALB/c (H-2^d) and CBA/H (H-2^k) mice were more efficient stimulators for H-2-matched T cells than those from C57BL/6 (H-2^b), B10.D2 (H-2^d) and B10.BR (H-2^k) mice. This effect shown by strains of C57BL background was

apparently manifested by suppression of T cell proliferation at the APC numbers optimal for maximum reactivity in other mouse strains. The finding that responses to VKV 1024, which encompasses the NS3 to NS5 region of the Kunjin genome, were always lower than those against VKV 1022 encoding mainly NS5 and a portion of NS4B, presumably reflects the differences in the processing requirements of these two proteins.

During the course of these investigations, suppression of T cell proliferation was observed when high numbers of macrophages and hyper-immune B cells were used as APC. Apparently, this suppression was not due to prostaglandins released by activated macrophages or exhaustion of nutrients from the culture medium. The contribution of MHC and the nominal antigen in causing this suppression of T cell proliferation was assessed. Thioglycollate-induced macrophages from CBA/H ($H\text{-}2^k$) mice which normally express low levels of MHC class II antigens, showed a direct dose response with alloreactive, $I\text{-}A^k$-specific T cells. In contrast, prior treatment of these APC for 1h with heat-killed *Listeria*, to maintain or upregulate MHC class II levels, resulted in an inverse dose response, with the highest T cell proliferation occurring at S:R ratio of 0.25 and profound suppression at a S:R ratio of 1 or 2. Addition of anti-Ia^b antibodies (or their Fab fragments) to the cultures at high APC numbers showed a significant improvement in the proliferative responses, indicating that high levels of MHC class II antigens contributed to suppression of T cell proliferation. The reversal of high APC number-induced suppression was achieved by reducing the concentrations of WNV antigen. These observations support the concept that the suppression of T cell proliferation observed

at high S:R ratios was due to supra-optimal concentration of ligand on APC. Possible implications of this phenomenon in relation to T cell tolerance are discussed.

LIST OF ABBREVIATIONS

2-ME	2-mercaptoethanol
antibiotics	Penicillin G 100 U/ml, Streptomycin 200 mg/ml and Neomycin 125 mg/ml.
APC	Antigen-presenting cell(s)
Con A	Concanavalin A
cpm	counts per minute
DC	Dendritic cell(s)
DMEM	Dulbecco's Modified Eagle's Medium.
DTH	Delayed-type hypersensitivity
EMEM	Eagle's Minimal Essential Medium.
ER	Endoplasmic reticulum
FACS	Fluorescence Activated Cell Sorter.
FcR	Receptors for immunoglobulin Fc piece
FCS	Foetal Calf Serum
h	hour
HAU	haemagglutinating units
Hepes	N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid
hkLM	Heat-killed *Listeria monocytogenes*
i.p.	intraperitoneal
i.v.	intravenous.
IFN-γ	Interferon gamma
IL-1	Interleukin 1
IL-2	Interleukin 2
IL-4	Interleukin 4
IL-6	Interleukin 6

KLH	Keyhole limpet haemocyanin
LM	*Listeria monocytogenes*-induced
LPS	Lipopolysaccharide
MAb	Monoclonal antibodies
MHC	Major Histocompatibility Complex
µl	microlitre
MLR	Mixed lymphocyte reaction
NK	Natural killer
NMS	Normal mouse serum
PBL	Peripheral blood leukocytes
PFC	Plaque forming cells
PG	Prostaglandins
pfu	plaque forming units
PMA	Phorbol myristate acetate
s.c.	subcutaneous
S.E.M.	Standard error of the mean
S:R ratios	Stimulator to responder ratios
SDC	Splenic Dendritic Cell(s)
SRBC	Sheep red blood cells
Tc cells/CTL	Cytotolytic T lymphocytes
TcR	T cell receptor
TH	Thioglycollate-induced
Th	Helper T lymphocytes
TNP	Trinitrophenol
UV	Ultra-violet
W/cm^2	Watts per sq. cm.

CHAPTER 1

GENERAL INTRODUCTION

CHAPTER 1

Contents

PREAMBLE

Macromolecular antigens usually express a large number of antigenic epitopes that dictate their specificity. However, only a limited number of the potential antigenic sites are important for immunogenicity, namely, immunodominant ones. The humoral and cellular arms of the adaptive immune system, which are mediated by B and T lymphocytes respectively, differ fundamentally in the way in which they recognize antigens. Antigen-binding B cells recognize conformation-dependent determinants on protein and carbohydrate antigens via surface immunoglobulin. In contrast, T cells, through their antigen-specific receptor, recognize a bimolecular ligand or "epitope", composed of a self-Major Histocompatibility Complex (MHC) molecule complexed with a small peptide, usually of 6 to 20 amino acids. This requirement, known as MHC restriction, ensures that T cell activation occurs only in an appropriate cellular context. Native antigens are modified by antigen presenting cells (APC) or target cells to produce peptides that can subsequently interact with MHC. Antigen-specific T cell activation thus results from binding of the TcR to a complex involving a peptide derived from the nominal antigen and class I or class II MHC molecules. For the purpose of discussion in this thesis the term "epitope" will be used to denote the portion of this complex bound by TcR involved in proliferative T cell responses.

Important regulatory cells of the immune system which recognize antigenic peptides in association with class II MHC

antigens are the helper/inducer class (Th cells) with the CD4+ (L3T4+) phenotype. Most of the effects of Th cells are mediated by the production of pleiotropic factors, called lymphokines, that influence a variety of functions such as activation, growth and differentiation of the cells of the immune system, including Th cells themselves. Lymphokine production is an induced property of activated T cells which depends upon TcR-mediated T cell activation. Since the TcR binds to a complex of antigenic peptide and MHC molecule, the antigen recognition event necessarily occurs at the surface of APC that express appropriate MHC molecules. Signal transduction subsequently leads to the secretion of lymphokines in the local environment. The specificity of the TcR ensures that the products of T cell activation are focussed upon the cells which present peptides, or other cells in the immediate environment. Thus, in the context of viral infections, Th cells participate in potentially protective immune responses by enhancing the production of virus neutralizing antibodies by B cells, by producing lymphokines such as interferon gamma (IFN-γ) that directly inhibit viral replication, and, in some cases by killing infected cells through the recognition of viral peptide-MHC class II complexes (Kaplan *et al.*, 1984).

The second major class of T cells that recognize peptide antigens in association with class I MHC antigens are CD8+ (Lyt-2+) T cells, which predominantly exhibit cytolytic effector function (Tc cells). These cells provide an efficient way for the immune system to eliminate viruses by killing infected cells before replication and synthesis of new virus particles is complete. Recently, these cells have been shown to secrete a

variety of lymphokines which may also contribute directly or indirectly to antiviral mechanisms.

In order to understand the specificity of the TcR, and thus, the mechanisms by which T cells carry out the process of self-non-self discrimination, it is necessary to review the cellular and molecular basis of antigen recognition.

1. Molecules of the immune system involved in T cell recognition:

1.1. Molecules present on T cells:

1.1.1. The T cell receptor α-β and γ-δ (TcR α-β, TcR γ-δ): The TcR of T cells was first identified with "anticlonotypic" monoclonal antibodies (MAb) raised against specific determinants on cloned T cells (Kappler *et al.*, 1981). The observation that these MAb bound only to the clones against which they were raised was the first direct indication that this structure was clonally distributed, similar to the surface immunoglobulin on B cells. By using such techniques (anticlonotypic MAb) it has been shown that, a) the putative human T cell receptor is a heterodimer consisting of two disulphide-bonded subunits, 49-51,000 (α) and 43,000 (β) daltons in molecular weight, which is non-covalently associated with a molecule T3, 20,000 daltons in molecular weight (Meuer *et al.*, 1983; Acuto *et al.*, 1983), and, b) the putative mouse T cell receptor is a heterodimer consisting of two disulphide-bonded subunits both 40-43,000 daltons in molecular weight (Allison *et al.*, 1982; Kappler *et al.*, 1983; McIntyre and Allison, 1983), containing regions of constant and variable structure. More recently the subtractive cDNA hybridization approach has revealed the sequences of the genes encoding for human and mouse TcR

(Hedrick *et al.*, 1984 a, b; Davis *et al.*, 1985 and Yanagi *et al.*, 1984; Chien *et al.*, 1984; Saito *et al.*, 1984). These studies have shown that there are separate variable (V), diversity (D), joining (J) and constant (C) region gene segments and, like immunoglobulin genes, these genes undergo somatic rearrangements (Chien *et al.*, 1984; Hedrick *et al.*, 1984). These α–β TcR are used for MHC class I and class II-restricted recognition by $CD8^+$ and $CD4^+$ T cells, respectively.

During the search for a rearranging gene encoding the TcR α component of TcR α β-heterodimer, TcR γ was identified by Tonegawa and colleagues (Saito *et al.*, 1984). Subsequently, a fourth rearranging gene, termed TcR δ, was discovered (Born *et al.*, 1987). A striking feature of the TcRγ and TcRδ loci is the relative small numbers of Vγ, Jγ and Vδ and Jδ (Strominger, 1989) segments that occur. In the mouse, there are about 100 α chain V gene segments, 25 for β, 7 for γ and 10 for δ. There are 2 D segment genes for β and γ chains, whereas J genes number 50 for α, 12 for β, 2 for γ and 2 for δ. Diversity of the TcR is generated by random rearrangements of gene segments. This means that a Vα gene segment joins randomly with any Jα segment and a C segment. In addition, diversity is also generated by, 1) imprecision in the joining of various gene segments resulting in deletion of nucleotides from the ends of the V, D and J segments (junctional diversity), 2) by the random additions of nucleotides in "N-region" junctional regions in both α and β chains (N region diversity), and, 3) by the use of multiple translational reading frames in the D region gene segments. Unlike immunoglobulins, diversity of TcR chains has not yet been shown to occur by somatic hypermutations.

1.1.2 TcR γ-δ cells: The majority of the murine T cells from the skin and in the gastrointestinal tract express TcRγ δ (Raulet, 1989). The ligand requirement for TcR γ δ is currently a matter of intense investigation. Although the TcRγ δ-expressing Tc cells recognized a class I MHC antigen, it appeared to be a non-classical transplantation antigen encoded in the TL region of the murine MHC (Bluestone *et al.*, 1988). Their role in surveillance of epithelial surfaces by recognizing yet uncharacterized class I-like gene products that map outside the classical class I and class II MHC gene complex, has been postulated (Janeway, 1988). The finding that TcRγ δ cells are capable of producing a variety of lymphokines including IL-2, IL-3, IL-4, IFN-γ and GM-CSF (granulocyte macrophage colony stimulating factor), suggests that these cells may exhibit multiple functions. In addition, their role in IgA secretion has been delineated (Bluestone and Matis, 1989).

1.1.3. CD3: The TcR (α-β and γ-δ) are covalently associated with an invariant membrane complex termed CD3. To date, four CD3 proteins have been identified (CD3-γ, CD3-δ, CD3-ε, and CD3-ζ) (Samelson *et al.*, 1985) on murine T cells. All the CD3 proteins have large intracytoplasmic portions. Cross-linking of CD3 mimics antigen-MHC stimulation of T cells. It is generally accepted that the CD3 complex is involved in signal transduction that is initiated when the TcR binds to the peptide-MHC complex.

1.1.4. CD4 and CD8 molecules: CD4 and CD8 are non-polymorphic members of the immunoglobulin gene superfamily which are expressed on the surface of functionally distinct populations of T

lymphocytes. These molecules are also known respectively as T4/Leu 3 and T8/Leu 2 in human, and as L3T4 and Lyt-2 in mouse.

The human CD4 molecule is a 55 kd glycoprotein with four extracellular domains (Littman, 1987), whereas the murine CD8 exists as a heterodimer consisting of two disulphide-linked subunits, Lyt 2(α), 38 kd, and Lyt 3(β) 30 kd.

CD4 was initially described as a phenotypic marker for T lymphocytes (Reinherz *et al.*, 1979). Direct proof that MHC class II is the ligand for CD4 has come from binding studies in which MHC class II^+ but not MHC class II^- B cells were shown to be capable of binding to the cell lines transfected with and expressing the human CD4 molecule (Doyle and Strominger, 1987). Both anti-CD4 and anti-class II MHC antibodies inhibited this binding. In the case of CD8 molecules, Swain *et al* (1981) demonstrated that addition of anti-Lyt-2 antibodies to *in vitro* cultures blocked the generation of cytotoxic respones against allogeneic class I MHC antigens but had no effect when these responses were specific for class II MHC antigens. Further evidence that the ligand for the CD8 molecule is MHC class I protein was obtained by Dembic *et al* (1987). These authors introduced the TcR α-β chain genes from CD8-positive, H-$2D^d$-restricted cytolytic T lymphocytes (CTL) specific for the antigen, fluorescein (FL), into CD8-negative recipient cells. It was observed that CD8-negative transfectant showed enhanced lysis of FL-conjugated lymphoblasts only after supertransfecting it with CD8 gene. These experiments established that the expression of CD8 molecules facilitates the interaction of CTL with their targets.

1.1.5. The CD2 receptor: CD2 [T 11, Leu 5, LFA-2, the sheep red blood cell (SRBC) receptor] is a non-polymorphic glycoprotein of 40 to 50 kd expressed on all human thymocytes and peripheral T cells, including the large granular lymphocytes and most natural killer (NK) cells (Howard *et al.*, 1981). Human CD2 mediates rosetting of T cells to SRBC (Krensky *et al.*, 1984). A natural ligand for CD2 has recently been found to be lymphocyte function-associated antigen-3 (LFA-3), a widely distributed glycoprotein of 55-70 kd (Shaw *et al.*, 1986). MAb directed against this protein inhibit the same range of T lymphocyte functions as anti-CD2 MAb, but this inhibition is by binding to the target or stimulator cell, not to the T cell (Shaw *et al.*, 1986). In addition, CD2 binding to LFA-3 mediates CTL-target cell conjugate formation (Shaw *et al.*, 1986). By expressing cDNA encoding the human CD2 in a murine T cell hybridoma with a particular antigen-specificity, Bierer *et al* (1988) showed that expression of CD2 molecule greatly enhanced T cell responsiveness to antigen, particularly in weak antigen-driven responses. The significance of this molecule in the activation of murine T cells is not known.

1.2. Molecules present on APC:

1.2.1. The murine major histocompatibility complex (MHC): The murine major histocompatibility gene complex designated *H-2*, is situated on chromosome 17 and spans 1-2 centimorgans of DNA distal to the centromere, where one centimorgan is equivalent to approximately 2000 kilobases. It can be broadly divided into the *K, I, S* and *D* regions (Klein, 1975; Snell *et al.*, 1976) which encode three classes of glycoproteins, the class I, class II and class III

molecules. Class I and class II molecules are integral membrane glycoproteins some of which constitute the histocompatibility antigens while class III molecules are serum components of the complement system (encoded by genes within the S region) and will not be discussed here.

1.2.1.A. MHC Class I antigens: The *K* and *D* regions of the murine MHC complex contain genes encoding class I molecules which are highly polymorphic, while the class I genes of the *Qa* and *Tla* regions show very little polymorphism. To date, approximately 50 class I (*K* and *D*) alleles have been identified.

The class I antigens constitute a single class structurally but functionally they fall into two groups.

The classical transplantation antigens are encoded by genes within the *K* and *D* regions. The *K* region contains genes encoding two molecules while D, L and possibly M and R antigens are encoded by genes within the *D* region. The class I transplantation antigens, especially K and D antigens, are found on virtually all somatic cells. These antigens have been shown to be the primary antigens recognized in tissue-graft rejection and to be restriction elements in the lysis by T cells of virally infected and chemically modified cells, (Zinkernagel and Doherty, 1979).

The antigens encoded within the *Qa* and *Tla* regions (distal to the *H-2D* region) which are believed to be lymphocyte differentiation antigens (Robertson, 1982).

1.2.1.B. Structure of MHC Class I antigens: The class I antigens consist of two chains, a 45,000 daltons heavy chain which is non-covalently associated with an 11,500 daltons light chain. The heavy chain is glycosylated, polymorphic, and is encoded within *H-2K, D* and *Qa* and *Tla* regions, while the light chain, β2-microglobulin is non-glycosylated, displays limited polymorphism and is encoded by a gene on chromosome 2. The heavy chain, approximately 346 amino acid residues in length, is divided into five domains, three external domains N (or α1), C1(or α2) and C2 (or α3) which are each about 90 residues in length, a transmembrane region, which consists of about 40 residues, and a cytoplasmic domain of about 30 residues in length. The third extracellular domain, C2, is highly conserved (Steinmetz *et al.*, 1982) and β2 microglobulin (β2m, 99 residues long) associates with this domain. The polymorphism of class I molecules appears to be clustered in three regions of the first two extracellular domains, N and C1 (Hood *et al.*, 1983). There is strong evidence that only the first and/or second external domains, N and C1 participate in the restricted recognition of antigen by the TcR (Ozato *et al.*, 1983; Stroynowski *et al.*, 1984; Allen *et al.*, 1984) and contain alloantigenic determinants (Yokoyama and Nathenson, 1983; Allen *et al.*, 1984).

The transmembrane domain serves to anchor the class I molecule in the cell membrane (Coligan *et al.*, 1981) and may function in the communication of signals to the cell interior (Coligan *et al.*, 1981).

Recently Bjorkman *et al* (1987 a,b) have produced an X-ray crystallographic model of an MHC class I molecule (HLA-A2) and from this have hypothesized its three-dimensional structure. The

molecule consists of four globular domains, the two membrane-proximal immunoglobulin-like domains, α3 and β2m, and the polymorphic α1 and α2 domains at the top (distal to the molecule). The latter two domains form a platform with a single eight-stranded β pleated sheet on top of which are two parallel α-helices. The net result is an elongated cleft, the sides formed by the α helices and the bottom formed by the β sheet structure. The cleft would seem to be able to accommodate antigenic peptides of the size known to be involved in T cell activation. Interestingly, the presence of electron dense material within the cleft was attributed to bound peptide although the nature of this material has not been directly demonstrated. These speculations are consistent with the principles of processed antigenic peptides binding to MHC as described in the Section 4.2.3.

1.2.1.C. MHC Class II antigens: The murine *I* (immune response) region, first recognized because of its role in the regulation of responses to synthetic and naturally occurring proteins, encodes MHC class II antigens and has been divided into the *I-A*, *I-B*, *I-J*, *I-E* and *I-C* subregions on the basis of serological analysis and functional studies (Shreffler *et al.*, 1976). The *I-A* subregion contains the A_α, A_β and E_β genes while the *I-E* subregion contains the E_α gene. Loci in the *I* region have been shown to control immunological reactions: e.g. (i) *I-A* and *I-E* loci encode allo-antigens which trigger graft rejection responses and may also serve as target antigens for CTL (Nabholz *et al.*, 1975; Wagner *et al.*, 1975); (ii) *I* region (*I-A* and *I-E*) encoded antigens are involved in the presentation of antigen to T cells which control the variation in immune response ability of various strains of

mice to some antigens (McDevitt *et al.*, 1982). This results from the fact that I-region encoded molecules control H-2 restricted interactions of Th cells with B cells (Katz *et al.*, 1975) and serve as restriction elements for T cells mediating delayed type hypersensitivity (DTH) (Miller *et al.*, 1975).

Class II MHC antigens are more limited in their cellular distribution than class I MHC antigens. They are found mainly on B cells, lymphoid dendritic cells (DC) and macrophages and variety of other cell types (reviewed in Chapter 3).

1.2.1.D. Structure of MHC Class II antigens: The two class II molecules encoded by genes within the *I-A* and *I-E* subregions are heterodimers composed of α and β chains which are non-covalently associated. The α chain is between 33,000 and 35,000 daltons in molecular weight and contains two carbohydrate groups; the β chain is 28,000 to 31,000 daltons and contains only one carbohydrate group. Each class II polypeptide consists of two external domains, each approximately 90 amino acid residues long (α1 and α2 or β1 and β2), a transmembrane region of about 30 residues and extremely short cytoplasmic region of approximately 10-15 residues. The polymorphism in the class II polypeptides lies mainly in the α1 and β1 domains whereas the α2, β2, transmembrane and cytoplasmic domains are conserved (Kaufman *et al.*, 1983). The transmembrane domain is even more highly conserved than α2 and β2 domains (Benoist *et al.*, 1983).

The b haplotype of the mouse does not express *I-E* molecules. This has been shown to be due to a deletion in the region containing the promoter for the *Eα* gene. However *Eβ*

chains are synthesized and found in the cytoplasm of cells but not expressed on the cell surface (Jones *et al.*, 1981).

In addition, the α and β chains are intracellularly associated with a non-polymorphic invariant chain (Ii) (Jones *et al.*, 1978), which is encoded by a single gene (Yamamoto *et al.*, 1985). The association between the Ia α-β heterodimer and the Ii (molecular weight 31,000) begins in the endoplasmic reticulum (ER) where Ii is synthesized. Ii dissociates from the Ia heterodimer somewhere between the trans-Golgi compartments. The specific association of Ii and Ia during biosynthesis (Holt *et al.*, 1985) led to the suggestion that Ii has a role in intracellular transport of Ia (see Section 4.2.3.).

On the basis of similarities in the structure of class I and class II molecules (close association of N-terminal residues of α1 and β1 domains of class II, and those of α1 and α2 domains in class I molecules), Brown *et al* (1988) postulated that the attachment of the class I α1 domain to β2m could be viewed as a four domain model as for class II heterodimer. Thus the physical form (i.e. an oligopeptide located in a cleft formed by the two α helices) of the antigen presented to T cells is likely to be similar for class I and class II molecules.

2. Cellular basis of antigen recognition by T cells:

2.1. Ontogeny of T cells: It is now well known that T cells acquire their antigen receptors as well as their functional potential to distinguish self from non-self within the developing thymus. This brief review will deal with α-β T cells.

Early T cell markers, such as CD7 (defined in man) or murine PGP-1 present on the pluripotent stem cells in foetal yolk

sac, bone marrow and liver, may function as homing receptors for the prothymocytes that colonize the foetal thymus at day 14 in mouse or 8 to 9 weeks in man. The subsequent developmental stages occur in the absence of a further influx of cells (Ceredig *et al.*, 1982). It is now evident that the majority of TcR gene rearrangements occur within $CD4^-$ $CD8^-$ precursors that are in a stage of rapid division. Following successful rearrangement of first the β gene and then the α gene, these cells stop dividing and express low levels of their receptors (α-β heterodimer and CD3 complex) along with two cell surface proteins, CD4 and CD8.

The first thymocyte to express detectable α-β TcR appear to be those of $CD4^+8^+$ T sub-populations, which appear at day 16-17 of gestation in mice, followed by thymocytes of mature $CD4^+$ $CD8^-$ or $CD4^-CD8^+$ phenotype, which appear around day 18-19 of gestation.

In the $CD4^+CD8^+$ (double positive) stage, the α-β cells apparently undergo the selection events described below.

2.2. T cell selection in thymus:

2.2.1. Positive selection: According to this hypothesis, precursors of mature T cells have some degree of binding specificity for polymorphic regions of MHC molecules expressed in the thymus. Although the earlier functional studies by Bevan and Fink (1978) and Zinkernagel *et al* (1978) using radiation-induced bone marrow chimeras revealed that radio-resistant elements of the host were limiting the potential repertoire of T cells, two recent experimental approaches involving *in vivo* antibody blocking and TcR transgenic mice strongly emphasize the role of thymic epithelium in the positive selection.

Marusic-Galesi *et al* (1989) observed that mice treated with MAb against K^k during foetal development failed to develop K^k-restricted T cells, while other class I-restricted T cells were generated undisturbed. From similar studies involving class II MHC, Marrack *et al* (1988) suggested that thymocytes are selected to mature in the thymus by the ability of their α-β receptors to interact specifically with class II MHC molecules expressed in the organ; treatment with anti-I-A antibodies apparently interfered with the interaction of TcR and I-A. These experiments did not, however, directly address the question of whether the α-β TcR is involved in the selection process.

Experiments using transgenic mice however, established a close correlation between α-β TcR specificity and thymic MHC antigens. Evidence to show that TcRs specific for MHC class I antigens are expressed in $CD8^+$ mature T cells has been reported by Teh *et al* (1988). In the case of female transgenic mice, an elevated proportion of thymocytes expressing α-β TcR genes isolated from an H-2^b-restricted $CD4^-8^+$ T cell clone, specific for an as yet to be identified peptide from the male-specific antigen, H-Y, was observed. In order to determine whether the elevated proportion of $CD4^-8^+$ T cells was dependent upon the interaction of the transgenic receptor with polymorphic domains of thymic MHC antigens, these authors repopulated the thymuses of mice of different MHC haplotypes with T-cell-depleted bone marrow cells from transgenic mice. An elevated proportion of $CD4^-8^+$ cells resulted from the colonization of the H-2^b, but not the H-2^k thymus by α-β transgenic cells. In another study, Kisielow *et al* (1988b) transplanted bone marrow cells from H-2^b α-β transgenic C57BL/6 (K^b, D^b) female mice into lethally irradiated

B10.HTG (K^d, D^b), B10.D2(R107) (K^b, D^d), F1(C57BL/6 x DBA/2) (K^bK^d, D^bD^d) and B10.BR (K^k, D^k) female mice. The finding that a significantly lower ratios of $CD4^+8^-$ to $CD4^-8^+$ thymocytes only in D^b-expressing [C57BL/6, B10.HTG and F1(C57BL/6 x DBA/2)] recipients of α-β transgenic stem cells than those differing at the whole H-2 complex (B10.BR) strongly suggested that preferential differentiation toward $CD4^-8^+$ thymocytes occurred only when transgenic stem cells expressing α-β transgenic chain develop in the thymic environment expressing restricting D^b molecules.

Evidence to show that MHC class II-restricted transgenic TcR are found on $CD4^+$ T cells has come from the studies by Berg *et al* (1989). These authors used transgenic mice expressing a TcR specific for a fragment of pigeon cytochrome C, restricted by $I\text{-}E^k$ molecules. It was observed that $H\text{-}2^k$ α-β transgenic mice expressed a 10-fold higher proportion of cytochrome C-reactive T cells than $H\text{-}2^b$ transgenic mice. Further it was shown that the developmental block at the $CD4^+8^+$ stage in the $H\text{-}2^b$ mice could be reversed by expressing $I\text{-}E^k$ from a transgene expressed on thymic cortical epithelial cells.

These observations indicate that the specific interaction of the TcR on immature thymocytes with thymic epithelial MHC antigens determines the differentiation of $CD4^+8^+$ thymocytes into single positive mature T cells. Because TcRα-β-expressing cells do not divide significantly in the thymus unless they bind to MHC antigens on epithelial cells, von Boehmer (1986) suggested that positive selection could be viewed as rescue from programmed cell death and depending on whether the

TcRα-β binds to class I or class II MHC antigens the rescued cell will be of the $CD4^-8^+$ or $CD4^+8^-$ phenotype (von Boehmer *et al.*, 1989).

Some variants of positive selection models proposed that the selection process does not involve *naked* MHC molecules, but rather thymic MHC complexed to a collection of unique self-antigens (termed as minor H antigens, Blanden and Ashman, 1985) expressed on the thymic stromal cells involved in positive selection but not outside the thymus. Marrack *et al* (1988) have explicitly proposed that thymic epithelium produces an epithelial-specific set of "educating peptides" which mimic the universe of potential foreign antigens. Further, negative selection (see the following Section) was proposed to be mediated by bone marrow-derived elements displaying a non-overlapping set of "deleting peptides" on their MHC which ensured deletion of T cells reactive to self antigens expressed outside the thymus. Kourilsky and Claverie (1989) argued that for the observed diversity of the T cell repertoire, thymic epithelial cells must present a large variety of erroneous self-peptides (due to errors in gene expression) which could be the major source of epitope diversity on these cells.

In addition to qualitative explanations of positive selection outlined above, there are quantitative models (see next section).

2.2.2. Negative selection: Negative selection was initially proposed to explain how the developing immune system learns to distinguish self from non-self, i.e., to react to foreign antigens yet be tolerant to self-antigens. Three general mechanisms have been proposed to account for self tolerance, 1) physical

elimination of self-reactive T cell clones in the thymus during ontogeny (clonal deletion), 2) self reactive T cells that do mature and enter the periphery could be functionally inhibited by other T cells (suppression/immunoregulation), and, 3) when self-reactive T cells encounter the antigen they recognize, they could be functionally inactivated (clonal anergy, discussed in Chapter 6).

Convincing evidence for intrathymic clonal deletion has been provided by the studies of Kappler *et al* (1987a,b). These authors identified MAb KJ23 that recognized a murine T cell Vβ segment (Vβ17a) that conferred a high probability of T cell reactivity with the I-E molecule. It was demonstrated that in mouse strains that lacked I-E MHC molecules, and so have no reason to be tolerant of I-E, 4 to 14% of splenic or lymph node T cells reacted with the antibody. In contrast, peripheral T cells from I-E$^+$ mice were only 0.1% positive. However, the results were quite different in the thymus. The immature thymic T cells of I-E$^+$ and I-E$^-$ mouse strains had eqivalent numbers of Vβ17a$^+$ cells. Thus when I-E was present, it apparently caused the elimination of anti-I-E (anti-self) T cells in the thymus during their maturation. Since low density KJ23$^+$ cells were detected in thymic cortex but not in medulla of I-E$^+$ mice, it was suggested that deletion of high density KJ23$^+$ cells occurs mainly in the medulla, i.e. the site of bone marrow-derived macrophages and DC, and therefore these two populations could be responsible for tolerance induction (Sprent *et al.*, 1988). In another study, Fowlkes *et al* (1988) used C57BR mice, which are both I-E$^+$ and possess a functional Vβ17a gene that encodes for the TcR recognizing I-E molecules. Reconstitution of irradiated C57BR

mice with syngeneic bone marrow, followed by anti-CD4 MAb treatment of such chimeric mice, resulted in, 1) elimination of $CD4^+8^-$ cells, and, 2) allowed the appearance of $CD4^-8^+$ cells expressing $V\beta17a^+$ thereby indicating that CD4 blockade specifically interfered with clonal deletion of $V\beta17a^+$, I-E reactive thymocytes at a $CD4^+8^+$ precursor stage. These experiments indicated that clonal deletion required the participation of the accessory molecule CD4 and that deletion occurred at the double positive stage. Similar results were obtained with Vβ6 expression in Mls^a mice treated *in vivo* with anti-CD4 MAb (MacDonald *et al.*, 1988).

Evidence for deletion of class I MHC-restricted TcRs came from the construction of transgenic mice expressing both α and β TcR chains from a CTL clone reactive with the male transplantation antigen-H-Y+H-2^b (Kisielow *et al.*, 1988a). Female transgenic mice (which were not tolerant to H-Y) expressed anti-H-Y transgenic receptors in a high proportion of T cells. Most peripheral T cells from such females could be stimulated to proliferate by male, H-Y-positive stimulator cells. In contrast, peripheral T cells expressing transgenic anti-H-Y TcR from male transgenic mice expressed low levels of CD8. In the thymus, male transgenic mice had 10-fold fewer cells than transgenic females, but at the $CD4^-8^-$ precursor stage these cells were present in normal amounts. Thus a major mechanism of immunologic self tolerance appears to be deletion in the thymus of clones of $CD4^+CD8^+$ T cells with α-β receptors specific for complexes of peptides derived from self proteins bound to self MHC molecules on the surface of bone marrow-derived DC. Low CD8 cells expressing α-β anti-H-Y TcR escaped deletion however,

presumably because high CD8 levels were required for the deletion process.

Sprent *et al* (1988) suggested the following quantitative scheme for T cell differentiation. Early T cells bind to MHC molecules on cortical epithelium with varying binding affinities. These populations undergo positive selection and make their way to the cortico-medullary junction. Here, bone marrow-derived macrophages and DC delete high affinity T cells (negative selection) but allow cells with low-intermediate affinity to exit from thymus to form the post-thymic repertoire of mature T cells.

Two recent hypotheses address the signalling requirements for T cell selection in the thymus. Sprent *et al* (1988) suggested that cortical epithelium generally releases positive signals because TcR density of early thymocytes is too low for high avidity binding. TcR density increases as the T cells move to the medulla, with the result that some of the cells acquire increased binding avidity. Contact of these cells with macrophages/DC in the medulla elicits negative signals and the cells are deleted, the remainder of the cells, i.e., cells with lower avidity, receive the same type of positive (protective) signal encountered in the cortex and are allowed to survive.

Blanden *et al* (1987) argued that T cell self tolerance is a quantitative phenomenon determined in part by self ligand concentration on selecting elements in the thymus and peripheral tissues and not simply related to the affinity between receptor and antigen. Assuming that the total signal strength required for T cell activation is a function of number of receptor/ligand complexes (Blanden *et al.*, 1987), for positive selection in the

thymus cortex it can be proposed that thymic epithelium expresses high MHC (ligand) concentrations so that pre-T cells with even low affinity for self MHC are saved from programmed cell death and stimulated to proliferate. Alternatively, or additionally, the ligand strength threshold required for positive selection could be low. Subsequently, the developing T cells are negatively selected for self tolerance during their passage through the cortico-medullary junctions, where they are confronted with bone marrow-derived macrophages and DC. Rationally the ligand concentrations, TcR concentrations, CD4 and CD8 concentrations, affinity of TcR for ligand and ligand strength threshold for negative selection should ensure that T cells emerge from the thymus only if they display above parameters which render them incapable of being activated by self ligands on self APC in the extrathymic environment. However, evidence to be presented in this thesis, together with previously published data (Matis *et al.*, 1983; Lamb *et al.*, 1983) indicate that peripheral T cells can be rendered unresponsive or anergic by high ligand concentrations, possibly an important peripheral mechanism maintaining self tolerance.

3. Cellular basis of antigen presentation:

3.1. Types of APC:

3.1.1. Macrophages: Murine macrophages express class I MHC antigens, FcR, C3R and a macrophage-specific cell surface marker defined by the MAb F4/80 (Hume *et al.*, 1983). The expression of class II antigens is limited to a sub-population of macrophages, the percentage of which varies in different tissues (Beller *et al.*, 1980). Class II MHC antigens can be induced on macrophages by

immune stimuli such as infection with *Listeria monocytogenes* (Beller *et al.*, 1980; Beller and Unanue, 1981) or by the deliberate addition of IFN-γ (Beller, 1984). Inflammatory stimuli such as thioglycollate broth, peptone or mineral oil, which increase the number of macrophages at the site of injection, do not induce class II MHC antigens on macrophages (Beller *et al.*, 1980).

Macrophages are highly motile and have potent phagocytic ability which can be enhanced by specific antibody, via FcR (reviewed by Unanue, 1981). They also apparently have the capacity to "process" antigen, but the biochemical mechanisms involved in processing are not fully elucidated. When macrophages are incubated with *Listeria* or ovalbumin, binding (a passive process which can occur at 4^0 C) of the antigens occurs rapidly (approximately 5 min) (Ziegler and Unanue, 1981). However, there appears to be a delay of 30-60 min between initial binding of antigen and its presentation to T cells (Calderon and Unanue, 1974; Ziegler and Unanue, 1981,1982). If macrophages were fixed (with paraformaldehyde or gluteraldehyde) immediately after antigen (soluble proteins and *Listeria*) binding, they could not stimulate T cell responses but could do so if fixed 30-60 min after antigen uptake (Ziegler and Unanue 1981). Further analysis showed that this critical 30-60 min incubation period involves a temperature-dependent, energy-requiring process, i.e., following antigen uptake, if macrophages were held at 4^0 C, or incubated at 37^0 C in the presence of a metabolic-energy inhibitor (e.g. sodium azide) they did not stimulate a T cell response. These data suggested that antigen processing is essential for presentation of protein and bacterial (*Listeria*) antigens to T cells and is an active process.

Finally, macrophages have been shown to secrete IL-1 or lymphocyte-activating factor (LAF, Unanue, 1981). In addition, several other cell types such as B cells, fibroblasts, keratinocytes, Langerhans cells, endothelial cells and NK cells also secrete IL-1. Two distinct, but structurally related molecules have been cloned (IL-1α and IL-1β). IL-1 is required for normal T cell development in the thymus (Mizel and Farrar, 1979), however its requirement in antigen-induced activation of mature T cells appears to be limited only to a sub-population of T cells, namely, TH2 (see Section 7). This cytokine also acts synergistically with another lymphokine, IL-6, which is also produced by macrophages, in the induction of T cell proliferation. In addition to its function as a pyrogen, inducing significant elevation of body temperature, IL-1 stimulates arachidonic acid metabolism (prostaglandin production) and the secretion of inflammatory proteins (Unanue, 1981).

3.1.2. B cells: B lymphocytes are distinguished by their expression of surface immunoglobulin (sIg) molecules which represent their antigen receptors. In addition to sIg, B cells express class I and class II MHC antigens, FcR, C3R and a variety of differentiation antigens such as Lyb1-7 antigens. With regard to antigen presentation, it is now widely accepted that activated B cells and some B cell tumors are able to present soluble antigens to antigen-specific primed T cells (Chesnut *et al.*, 1982; Frohman and Cowing, 1985; Krieger *et al.*, 1985).

While there have been many reports that resting B cells are poor APC for the presentation of soluble antigens to primed T cells (Chesnut *et al.*, 1982; Kakiuchi *et al.*, 1983; Krieger *et al.*,

1986) and are also poor stimulators of unprimed allogeneic T cells, others have claimed that resting B cells are efficient stimulators provided treatment with high dose γ-irradiation (above 1000 rads) is avoided (Ashwell *et al.*, 1984).

In contrast with the current controversy surrounding the stimulatory capacity of resting B cells, a general consensus is that activated B cells are much more potent APC than resting B cells. Current evidence suggests that B cells play a major role as APC in primary T cell responses to soluble antigens in lymph nodes *in vivo* (Ron *et al.*, 1981; Ron and Sprent, 1987). These authors reported that mice depleted of B cells from birth by repeated injections of antibody directed against the μ-chain of IgM failed to undergo T cell priming to soluble antigen in lymph nodes (as evidenced by *in vitro* proliferation), and that the defect was overcome by sub-cutaneous (s.c.) injections of purified lymph node B cells from normal mice before the injection of antigen. In another experiment these authors performed *in vivo* plaque forming assays to assess whether poor priming of T cells had adversely affected their helper function (i.e. T-B interaction). Irradiated mice (mice depleted of B and T cells, but presumably containing DC and macrophages) when reconstituted with normal unprimed T cells (without s.c. injection of B cells) and subsequently challenged with antigen, showed poor proliferation responses (like anti-μ-treated mice) but gave significantly higher responses in the helper assay than unprimed T cells, although much lower than T cells primed in lymph nodes of normal mice. This means that the transferred T cells had undergone some degree of sensitization in the absence of B cell APC. These authors proposed that the activation of resting T cells in the

lymph node is controlled by non-B APC, and that main role of B cells is to induce clonal expansion of activated T cells.

3.1.2.A. Cellular basis of inefficient antigen-presenting by resting B cells: Several explanations have been put forth to account for inefficient antigen-presentation by resting B cells. The finding that the α chain of class II antigens from resting B cells was more heavily sialated than those from non-B APC (Cullen *et al.*, 1981), lead to the demonstration by Cowing and Chapdelaine (1983) and Frohman and Cowing (1985) that neuraminidase treatment of resting B cells increased their T cell stimulating capacity in primary and secondary T cell responses to alloantigens. However, subsequently it was shown that neuraminidase-treated T cells showed a similar increase in response which was attributed to the alteration of net surface charges, thus facilitating B and T cell interactions, rather than an effect on class II antigens (Krieger *et al.*, 1988). Further, when the class II antigens from resting and activated B cells were incorporated in artificial planar membranes they showed a similar capacity to present ovalbumin peptides to specific T cells, and this presentation was independent of neuraminidase treatment (Krieger *et al.*, 1988). Thus factors other than the quality of class II MHC must account for inefficient APC function of resting B cells.

3.1.2.B. Antigen-specific B cells: The first indication that cell surface Ig can confer a high potency advantage to antigen presenting B cells, came from the studies reported by Chesnut and Grey (1981). These authors showed that murine B cells could

stimulate the proliferation of rabbit immunoglobulin-specific T cells only when rabbit anti-mouse IgG (but not normal rabbit IgG) was used as an antigen. Subsequently, it was shown that this specific antigen was efficiently presented when added to B cells at 10,000-fold lower concentration than non-specific antigen (normal rabbit immunoglobulin) (Chesnut and Grey, 1986; Tony *et al.*, 1985; Tony and Parker, 1985). Lanzavecchia (1985) used an entirely antigen-specific system to show the efficiency of sIg in antigen uptake. He showed that EBV-transformed, tetanus-toxoid-specific (TT-specific) B cells present specific antigen to TT-specific T cell clones when exposed to 10^4-fold lower antigen concentration than that needed for EBV-transformed B cell lines not specific for TT. Only a small proportion of the total sIg molecules on B cells are necessary to take up the amount of antigen required for subsequent presentation to T cells (Lanzavecchia, 1987). This binding of antigen to sIg can be blocked by soluble antibodies that react with the same epitope. The high efficiency of antigen-presentation by these cells was explained on the basis of 2 observations, 1) the rapid internalization of antigen at 37^0 C by receptor-mediated endocytosis thus, delivering to the cell every h, 5-10 times the amount of antigen that is actually bound to sIg at any given time, and, 2) the long half life of processed antigen on B cells (24 h).

3.1.3. Lymphoid dendritic cells: DC are present in white pulp of spleen and lymph nodes (Steinman and Cohn, 1973) and are present in all the non-lymphoid tissues. Interdigitating cells present in T-dependent areas of lymphoid tissue (Veerman, 1974), veiled cells of afferent lymph (Drexhage *et al.*, 1979) and

indeterminate cells of skin (Hume *et al.*, 1983) probably belong to the same class as DC defined by *in vitro* properties after isolation from secondary lymphoid tissues. DC are large irregular cells with long stellate processes and are highly motile (Steinman and Cohn, 1973). DC do not proliferate rapidly but are constantly turning over in lymphoid organs at a rapid rate (more rapid than macrophages) (Steinman *et al.*, 1974). This suggests that they either have a short life span or emigrate to other sites rapidly.

DC do not express FcR, C3R, the murine macrophage-specific marker F4/80, Thy-1 antigen or sIg. They express a DC-specific marker defined by MAb, 33D1 (Nussenzweig *et al.*, 1982) and express high levels of class I and class II MHC antigens (Nussenzweig *et al.*, 1981).

DC have been shown to be strong stimulators of primary syngeneic and allogeneic mixed lymphocyte reactions (MLR) (Steinman and Witmer, 1978; Nussenzweig and Steinman, 1980; Steinman *et al.*, 1983) and to function as APC in the development of anti-trinitrophenol (TNP)-specific Tc cell responses (Nussenzweig *et al.*, 1980).

The question as to whether DC are capable of processing and/or presenting foreign antigens is contentious. They do not phagocytose particles and have weak pinocytic activity (Austyn, 1987). Furthermore, Koide and Steinman (1987) have shown that DC do not contain IL-1 message. They have been shown to function as APC in the development of influenza-specific Tc cell responses (Macatonia *et al.*, 1989) and to present soluble antigens such as keyhole limpet haemocyanin (KLH) and ovalbumin (Miyazaki and Osawa, 1983; Sunshine *et al.*, 1983; Guidos *et al.*, 1984). In another report it was shown that while DC presented

soluble mycobacterial antigen (purified protein derivative) more efficiently than macrophages, they were just as effective as macrophages in presenting whole mycobacteria.

Recently, Romani *et al* (1989) showed that splenic dendritic cells (SDC) and cultured epidermal Langerhans cells weakly present native myoglobin to myoglobin-specific T cell clones, but these APC were powerful stimulators of allogeneic T cells in primary MLR. Freshly isolated Langerhans cells, in contrast, were very active in presenting myoglobin to T cell clones but were weak stimulators of MLR. These results led them to suggest that DC in non-lymphoid tissues can act as sentinels for presenting antigens *in vivo*, their APC function develops in two stages. First antigens are captured and presented. Further handling of antigen is then downregulated while the cells acquire strong sensitizing activity for the activation of resting T cells.

DC can physically associate, or cluster, with T cells in a unique manner that is independent of antigen and MHC. In short term assays (Inaba and Steinman, 1986) clustering of DC with T cell blasts, irrespective of their specificity was observed at 37^0 C. In contrast, other APC cluster with T blasts only in the presence of specific antigen and relevant MHC, and this can occurr at 4^0 C. These authors suggested that DC-T cell clustering, which occurs in all primary responses, is essential for T cell activation.

It thus appears that DC have a definite role at least in the activation of resting T cells. In addition, their unique anatomic distribution and recirculation pattern (discussed in Chapter 3) may further contribute to fulfil their role as a predominant APC in the activation of resting T cells.

4. Antigen-processing and presentation:

Antigen processing encompasses the metabolic events that a protein antigen must undergo in or on the APC before it can be recognized by the T lymphocytes. Processing involves proteolytic degradation, denaturation, or modification of the antigen, complexing of antigenic fragments with class I or class II MHC molecules and the expression of such complexes on the surface of APC.

Although Tc and Th cells normally recognize antigens in association with class I and class II MHC antigens respectively, the distinction between MHC class I-and class II-restricted presentation cannot be made solely at the level of cell types expressing these molecules, because class II positive cells also express class I molecules. Recent data have suggested that this distinction may be made by the source of antigen; exogenously derived antigens are presented by class II molecules, whereas endogenously synthesized antigens are presented by class I molecules (Morrison *et al.*, 1986; Germain, 1986). Sub-cellular mechanisms are not yet fully understood, but in general, exogenously derived antigens require a passage through endosomes, whereas endogenously synthesized proteins are processed by a non-endosomal pathway that is not yet defined. The salient features of these pathways, and the steps involved in the endosomal pathway will be discussed in detail in the following sections.

4.1: Antigen-processing for MHC class I-restricted presentation:

Most class I-restricted T cells recognize antigens that have been synthesized by the cells that present them (Morrison *et al.*, 1986, 1988). Endogenously synthesized antigens fall into at least two classes, 1) those which are directed to the cell surface or secreted, and, 2) those which remain within the cytosol or the nucleus. The former category of antigens penetrate the ER and meet, at some stage, newly synthesized and/or recycling MHC molecules. The second category of antigens somehow cross a membrane and associate with MHC molecules by a process which is not fully understood. Both types of endogenous antigens are presented as illustrated by class I-mediated presentation of endogenously synthesized influenza nucleoprotein and haemagglutinin (Townsend *et al.*, 1988 a,b; Braciale *et al.*, 1987).

When a modified influenza haemagglutinin gene, engineered by deleting the signal sequence (Townsend *et al.*, 1986b) or the membrane anchor sequence (Braciale *et al.*, 1987), was used for transfecting target cells, many Tc cell clones directed against mouse cells expressing the wild type membrane-anchored protein still recognized cells expressing the modified gene, thereby indicating that viral antigens need not be expressed on the surface of infected cells for Tc cell recognition.

The viruses expressing fusion proteins cause their structural proteins to enter the cytosol of cell with which they fuse. Yewdell *et al* (1988) showed that heat-inactivated influenza virus which retains fusion activity, but is no longer able to replicate, sensitized target cells for lysis by Tc cells. Both internal (nucleoprotein and polymerase, PB1) and external

(haemagglutinin) proteins derived from virions were processed by cells for Tc cell recognition. This sensitization required the inactivation of viral neuraminidase activity. However, the inactivation of viral fusion activity by brief exposure of heated virus to pH 5 prevented target cell sensitization for lysis by Tc cells. These studies established that intact, structural viral proteins can be processed by target cells given that sufficient quantities are delivered to their cytoplasm.

Introduction of soluble proteins into the cytoplasm also sensitizes the target cells for Tc cell recognition. Moore *et al* (1988) obtained chicken ovalbumin (OVA)-specific Tc cell responses by immunizing mice with transfected cells which produced OVA endogenously. Target cells incubated with native OVA (pinocytosed OVA) were not sensitized for Tc cell recognition. However, when OVA was released into the cytosol, by osmotic lysis of pinosomes, it was processed and the resulting peptides associated efficiently with class I molecules. Recently, by using the antibiotic brefeldin, which blocks the transport of proteins out of the ER, it was shown that processed cytoplasmic antigens associate with class I molecules prior to their egress from the ER (Long, 1989).

4.2: Antigen-processing for MHC class II-restricted presentation:

The various stages of antigen processing can be defined as, 1) initial antigen interaction with an APC and uptake of antigen, 2) processing of antigens by as yet undefined enzymes, and, 3) the interaction of processed antigen with class II molecules.

4.2.1. Initial antigen interaction with APC and uptake:

Initial interaction between antigen and APC surface strongly influences the efficiency of processing (Snider and Segal, 1987; Lazavecchia, 1987; Manca *et al.*, 1988). These interactions can either be direct and specific (e.g. surface sIg of antigen-specific B cells) or mediated by interactions of antigen/antibody/complement complexes with surface C3R and FcR. Increased positive charge on the antigen also enhances processing, probably by increasing binding to the negatively charged cell surface (Apple *et al.*, 1988). The uptake of antigen is both time and concentration-dependent and may involve pinocytosis or phagocytosis.

Another potential pathway of antigen uptake is fluid phase pinocytosis. However its mechanism has not yet been clearly established. After the initial phase of endocytic adsorption of the protein on the plasma membrane of the cell, a pinocytic vacuole is formed by invagination of plasma membrane which subsequently travels towards lysosomes.

4.2.2. Processing of antigens by intracellular enzymes: There is now considerable evidence that at least one obligatory step in the processing of a variety of protein antigens is proteolysis, since antigen processing can be blocked by a variety of specific protease inhibitors. Recently Yoshikwa *et al* (1987) showed that the variability of antigen presetation by TNP-specific B cells treated with leucopeptin, a thiol protease inhibitor, is due to the usage of different proteases in the generation of specific epitopes recognized by the Th cells utilized. These authors suggested that

different groups of proteases are required during antigen processing, so that a particular specific antigenic epitope can be presented to and recognized by the TcR. Buus and Werdelin (1986) have examined the role of proteolysis in the presentation of antigens by guinea pig accessory cells by using benzyloxycarbonyl-phenylalanylalanine-diazomethyl-ketone (ZPADK), a group-specific inhibitor of 'cysteine proteinases' and pepstatin A, group-specific inhibitor of 'aspartic proteinases'. The results indicated that inhibition of cysteine proteinases of APC caused profound inhibition of both the proteolytic degradation and presentation of dinitrophenyl-poly-L-ysine. However the presentation of another synthetic antigen, a copolymer of L-glutamic acid and L-lysine was enhanced by the same inhibitor. These results indicate that, a) inhibitor may completely block the presentation if it inhibits a proteinase which is absolutely necessary for the production of the epitope-carrying fragment, b) the inhibitor may enhance the presentation of the epitope if it inhibits a proteinase which normally causes destruction of the fragment carrying the epitope.

A number of compounds have been shown to block class II MHC-restricted presentation by APC of endocytosed antigens. These include acidotropic agents (chloroquine and NH_4Cl; Ziegler and Unanue, 1982), inhibitors of membrane recycling (such as monensin), inhibitors of proteases (leucine peptidase and cysteine proteases); (Werdelin *et al.*, 1988), and cerulinin (which blocks posttranslational modifications of proteins by lipids; Falo *et al.*, 1987). These studies imply that low pH proteolysis of antigens within endosomes is one of the essential requirements for class II-restricted presentation. Recently, Guagliardi *et al*

(1990) defined the pathway for class II antigens by immunoelectron microscopy. They showed that the export pathway for class II MHC antigens and the antigen import pathway meet in a peripheral early endocytic compartment which has all the machinery for processing and presentation. These include, proteolytic enzymes (cathepsin B and D) and class II molecules en route to the cell surface.

The evidence to support a definitive role of the Ii, which associates with class II MHC molecules soon after their synthesis in the ER, has been investigated in recent studies. The oligomeric class II-Ii complex moves through the Golgi apparatus where the proteins are terminally glycosylated. Since the processing and release of class II proteins from Ii can be inhibited by the protease inhibitor, leupeptin, as well as by chloroquine and NH_4Cl, it has been suggested that it is an endosomal compartment in which dissociation of class II proteins from Ii occurs. This view was supported by Cresswell (1985) who showed that endocytosed transferrin receptor i.e. ferritin-neuraminidase conjugates, meet Ii and class II polypeptides in an early endosomal compartment. Recently Stockinger *et al* (1989) found that some class II-transfected fibroblasts could present antigen only if it was provided in fragmented form. These cells did not process/present native antigen. However, if class II-transfected fibroblasts were also transfected with the Ii chain gene (either Ii 32 or Ii 41), they efficiently stimulated T cells (Stockinger *et al.*, 1989). Further, chloroquine treatment of fibroblasts abrogated the presentation of native antigen but not of fragmented antigen. These studies demonstrated that Ii is required for appropriate

processing of antigen or for binding of processed antigen to class II protein.

The form of an antigen itself influences the processing requirements. On the basis of processing requirements of different antigens, Allen (1987) suggested 3 types of T cell determinants. Type I determinants are located on a portion of the peptide which has well segregated agretopes (MHC-interacting site) and epitopes (TcR interacting site; Katz *et al.*, 1983) and they do not require processing. For example, determinants on the carboxy portion of a chain of human fibrinogen do not require processing (as fixed or chloroquine-treated APC were able to present this protein to T cells (reviewed by Allan *et al.*, 1987). Lee *et al* (1988) mapped the T cell epitope on this molecule to Aα (551-571), which is located on the carboxy portion of the Aα chain. Since this portion had no defined secondary structure, these authors proposed that the direct association of this portion with I-E^k could be due to its conformational flexibility. Recognition of such a large molecule (340,000 molecular weight) without processing contrasts with the prevailing views about antigen processing. However, in this case it is possible that, 1) proteases associated with the T cell could degrade the antigen, or 2) a proportion of molecules in non-native state added to the surface of APC might have bound to Ia antigens. Type II determinants simply require the polypeptide chain to be unfolded for processing (e.g. lysozyme, ribonuclease, myoglobin) and Type III determinants are those which require proteolytic cleavage to meet the conformational flexibility nedded to form the correct Ia and T cell contact structure [ovalbumin (OVA), cytochrome C, insulin etc.].

In the pigeon cytochrome C system, the fragment of cytochrome containing residues 60-114 required processing, whereas simply removing six residues 60-65, abolished this processing requirement (Kovac and Schwartz, 1985). This was shown to be due to a proposed T cell contact residue (Lys 99) which was accessible only after processing

In addition, amino acid residues not included in the epitope influenced processing. For example, in the lysozyme system, Shastri *et al* (1986) found that the residues outside the minimal peptide determinant used, strongly influenced the antigenicity in H-2^b mice, which was thought to be due to promoting or inhibiting unfolding and cleavage of the peptide.

Thus, the determinants within a given protein together with the specificity of proteinases involved in antigen processing contribute to define the specificity of T cell responses.

4.2.3. Interaction of processed antigen with MHC molecules: The first experimental evidence in favour of a specific interaction between antigens and MHC came from the functional studies by Werdelin (1982) showing that antigens restricted by the same MHC molecules could compete for presentation at the level of APC. In an attempt to demonstrate that antigenic fragments could bind directly to MHC class II molecules, Babbitt *et al* (1985, 1986) performed equilibrium dialysis studies using affinity-purified MHC molecules and labeled synthetic peptides. It was demonstrated that HEL peptide 46 to 61 bound to I-A^k (from high responder strain, H-2^k) but not to I-A^d (from low responder strain, H-2^d). Similar results were obtained using OVA 323-339 (Buus *et al.*, 1986, 1987). These results suggested that it was

necessary for immunogenicity that peptides bind to the MHC restriction element and that only peptides which bind to MHC molecules can be recognized by T lymphocytes. However, it did not mean that all peptide-MHC complexes were immunogenic.

MHC molecules cannot distinguish between self and non-self peptides. Recent experiments using haemoglobin variants of the mouse have shown that self haemoglobin-Ia complexes permanently exist *in vivo* on APC (Lorenz and Allen, 1988). The general notion from such studies is that there is a diverse array of MHC-peptide complexes on cell surfaces, with peptides being derived from internal self-proteins (for class I) or from endocytosed self-proteins (for class II). The ability/inability of self peptides to bind MHC in relation to the mechanism of self-tolerance was studied by Babbitt *et al* (1986) who showed that the murine peptide analogue of HEL (52-61), which differs from HEL (52-61) only by single amino acid, can indeed bind to a murine I-A^k molecules but cannot stimulate a HEL (52-61)-specific murine T cell hybridoma. Similarly, Adorini *et al* (1988) showed that a synthetic peptide of mouse lysozyme, corresponding to residues 46-62, which binds to I-A^k although not immunogenic itself, effectively inhibited priming for T cell responses when injected with immunogenic HEL 46-61 or HEL 112-129. The latter generate responses restricted by I-A^k. In contrast, Lorenz and Allen (1988) showed that murine ribonuclease (RNase) peptide (43-56) did not bind to purified I-A^k and could not compete for the functional presentation of bovine RNase (43-56) to a specific T cell hybridoma, which led them to conclude that self/non-self discrimination can occur by at least two mechanisms. First, if a self-peptide cannot bind to

the class II molecule, then it could never be immunogenic for class II-restricted T cells. However, if the self peptide could bind to the class II molecule, then self-tolerance would need to occur either through functional or physical deletion of the potentially self-reactive T cells.

If self antigens compete with foreign antigens, then the question arises as to how an immune response could develop in the context of such a vast excess of self proteins. Recently, Lorenz *et al* (1990) addressed this question by examining the ability of normal mouse serum to inhibit the presentation of bovine RNase A that enters an APC through fluid phase pinocytosis and M-RNase (mannosylated RNase), which was taken up by APC through the mannose receptor. The results showed that the presentation of M-RNase was minimally reduced by competition with self proteins, whereas the presentation of non-M-RNase was almost entirely inhibited. These results indicate that receptor-mediated uptake of antigens can be a critical step in overcoming the competition with self peptides during the generation of immune responses *in vivo*.

The necessity of antigenic-MHC interactions for immunogenicity was further supported by competition experiments. Buus *et al* (1987) studied the binding of 12 different antigenic peptides to 4 different class II molecules. The binding was examined directly as well as by inhibition assay in which 12 unlabeled peptides were used to inhibit the binding of a labeled peptide known to bind to a particular MHC molecule. It was shown, 1) peptides that would bind to a particular MHC molecule would inhibit the binding of any other peptide to that molecule, thereby indicating that all peptides compete for the

same MHC-binding site i.e. an MHC molecule can bind only one peptide at one point in time, and, 2) binding or competition was always correlated with the known MHC specificity of immune responses to a given antigen.

These experiments support the "determinant selection" hypothesis (Barcinski and Rosenthal, 1978) in the sense that class II molecules somehow select the part of the antigen that will serve as a determinant for T cell recognition and can also explain the immune response category of a responding or non-responding genotype in some cases (Schwartz, 1986).

Thus the net result of antigen processing is salvation of potential epitope carrying fragments from total proteolytic degradation ("determinant protection", Werdelin, 1986) and their association with MHC class II antigens for TcR recognition. Minimum peptide length necessary for T cell stimulation was determined by using sets of overlapping synthetic peptides and testing their ability to stimulate T cells. A number of antigenic sites were identified in this way on proteins such as staphylococcal nuclease, cytochrome C, myoglobin, insulin and OVA (reviewed by Livingstone and Fathman, 1987). These studies indicate that T-cell epitopes depend upon peptides of approximately 6 to 20 amino acids. Further, the importance of a particular residue has been examined in such studies using peptides substituted with different residues at various positions. When the conformational preferences of polypeptide segments from moth and pigeon cytochrome C (residues 88-103 or 87-104 of the pigeon cytochrome C) were examined (Pincus *et al.*, 1985), the residues 88-91 and 94-98 from pigeon and moth, respectively, showed a strong tendency to form an α–helix, despite a

difference in sequences at the residues 88-89. From these studies a correlation between conformation (α-helix in this case) and biological activity was established.

Schwartz *et al* (1985) further analyzed the biophysical aspects of T cell responses to moth cytochrome C, with known TcR contact residues, 97-103. Addition of residues outside the epitope increased the antigenic potency which was thought to be due to the stabilization of the secondary structure in α-helical configuration. Thus the secondary structure of peptides may play a role in determining the potency of antigenic determinants involved in the activation of T cells. Several groups have suggested different models for predicting T cell epitopes by comparing the sequences of known epitopes. These models are based upon amphipathic conformation (Delisi and Berzofsky, 1985) and presence (or absence) of charged residues in a given peptide (Rothbard and Taylor, 1988). Although these models have predicted T cell epitopes in a variety of antigens, their generality is yet to be established.

5. Immune response gene (Ir) effects:

Ir genes control differences in the magnitude and fine specificity of an immune response as well as responsiveness or non-responsiveness to a given antigen. It is now well established that important Ir genes code for MHC molecules and are involved in MHC-restricted T cell interactions (reviewed by Schwartz, 1986). Several mechanisms have been proposed to account for the effect of class II MHC genes on T cell responses. These models can be grouped into 2 categories, antigen presentation models and repertoire selection models. At present there is no

substantial evidence to argue that one of these mechanisms of Ir gene-controlled non-responsiveness is dominant.

5.1. Antigen presentation models: These models hold that Ir genes function at the level of antigen processing and/or class II MHC-peptide interaction, and the non-responsiveness is due to an inability of APC to present antigen to T cells. Evidence for this came from the studies by Babbitt *et al* (1985) who showed that the binding affinity of Ia molecules for the processed antigen is obligatory for the presentation of the latter (discussed in detail in section 4.2.3.). The antigen-presentation models assume that the mature T cell repertoire may not differ between responder and non-responder strains. Such models cannot account for failure to respond to self peptide-MHC complexes which are known to exist (Lorenz and Allan, 1988; Buus *et al.*, 1988).

5.2. Repertoire selection models: Several different models have been postulated to account for repertoire selection. These are,

5.2.1. Limitations in the germline repertoire or "hole in the repertoire" model: According to this model, the degree of TcR diversity is not sufficient enough to generate a totipotent repertoire, which leads to the formation of "holes in the repertoire". A limited germline repertoire might preclude the formation of a combining site for a particular form of MHC molecule (Schwartz, 1986). This seems to be only a theoretical possibility because natural selection should eliminate individuals with inherent low diversity of T cells.

5.2.2. MHC-restricted tolerance model: An alternative effect of MHC molecules on the response phenotype could be due to selective differences in the T cell repertoire caused by MHC-

dependent selection mechanisms (Schwartz, 1978). For example, the T cell population is presumably physically or functionally purged of any cell that recognizes self-protein products in association with self MHC molecules. If these self-antigen-MHC complexes cross react with foreign antigen-self MHC complexes then the strain would be unresponsive to the foreign antigen. Mullbacher *et al* (1983) showed that presence of K^k in H-2 recombinants such as 2R (K^k D^b) and 4R(K^k D^b) or in F1 hybrids, greatly reduced the anti-vaccinia-D^b response; this was not the case with the K^b, K^d and K^q alleles. These authors proposed that the suppressive influence of K^k on anti-vaccinia-D^b Tc cell responses was as a result of self-tolerance in which K^k (possibly plus self peptide) cross-reacted with vaccinia + D^b.

Guillet *et al* (1987), in analyzing the response to a peptide (12-26), derived from bacteriophage lambda cI repressor protein, found that this peptide bound significantly to I-A^d, but much stronger to I-E^d, and yet the T cell response was restricted to I-A^d and not to I-E^d. The lack of I-E^d-restricted T cell responses in this case can be explained on the basis of self-tolerance, in which I-E^d (and possibly self peptide) cross-reacted with peptide (12-26) complexed with I-E^d.

5.2.3. Preferential generation of suppressor T cells: Definitive evidence for antigen-induced, Ia-restricted activation of suppressor cell came from the studies reported by Asanso and Hodes (1983). Lyt-1^+2^- T cells from parent A suppressed, in antigen-specific fashion, the responses generated by (A x B) F1 Th cells cooperating with parent A cells, but did not suppress responses by the same (A x B) F1 Th cell population cooperating

with parent B. Whether such phenomena have more widespread significance is unknown.

6. MHC-restricted T cell activation:

Following the presentation of an antigenic peptide in association with class II (or class I) MHC molecules, specific recognition by TcR leads to receptor-mediated signal transduction in T cells. This is one of the fundamental mechanisms in the transduction of a wide variety of extracellular signals into cellular responses. Various other ligands that bind to TcR such as MAbs against CD2, CD3 or calcium ionophores also induce phosphoinositide degradation and Ca^{2+} mobilization, although the precise role played by the latter in the activation of lymphokine encoding genes is not known. The process of T cell activation is accompanied by massive reorganization of cytoplasmic organelles and of cytoskeletal proteins inside the cell, and the TcR become concentrated in the region in contact with the APC (Kupfer *et al.*, 1987).

7. Heterogeneity of MHC class II-restricted T cells:

In past few years Th cells have been shown to secrete a variety of lymphokines, which are now collectively known as interleukins (ILs). Traditionally, $CD4^+$ T cells have been described as helper cells, although this term was originally defined by the ability of T cells to help a B cell to make specific antibody. Now we know that subsets of murine $CD4^+$ T cell lines (TH1 and TH2) produce distinct patterns of lymphokines (Mosmann *et al.*, 1986). TH1 cells produce IL-2 and IFN-γ, in

response to MHC-restricted antigen presentation or Concanavalin A (Con A), whereas TH2 cells produce IL-4, IL-5 and IL-6. Both the cell types produce IL-3. Janeway *et al* (1988) referred to TH1 as inflammatory T cells (T inf); and TH2 as helper T cells (Th). Mosmann *et al* (1986) further showed that these two subsets require different autocrine growth factors; IL-4 being a growth factor for TH2, (which functions only in the presence of IL-1) and IL-2 for TH1 cells. Since IL-4 is required for the production of IgG1 and IgE antibodies, (Vitetta *et al.*, 1985) the responses driven by TH2 cells are dominated by the antibodies of these isotypes, and these responses can be inhibited both *in vivo* and *in vitro* by anti-IL-4 antibodies (Snapper and Paul, 1987). However, this functional dichotomy of class II-restricted T cells does not hold all the time. Several short-term human and murine T cell clones showed a marked deviation from TH1/TH2 classification (reviewed by Kelso, 1990). Therefore it seems that peripheral T cells are not precommitted to a particular lymphokine secretion pattern. The question as to whether there is a selective activation of TH1 and TH2 subsets during *in vivo* priming is rather contentious. The inferences derived from several *in vitro* studies strongly suggest the possibility of *in vivo* occurence of these subsets. These are,

1) Splenic dendritic cells have been shown to present antigen to T cells that can proliferate *in vitro* in response to antigen (TH1) but not to functional B cell helpers; whereas splenic and peritoneal macrophages can present antigens to both TH1 and TH2 T cells (Ramila *et al.*, 1985).

2) Tite *et al* (1987) showed that mice expressing I-A^s make a strong *in vitro* proliferative response to human basement

membrane collagen type IV. In contrast, when serum antibody production was measured, only mice expressing I-A^s failed to make an effective antibody response, whereas (I-A^s x I-A^{non-s}) F1 showed a strong T cell proliferative response, but gave lower antibody responses than I-A^{non-s}. Janeway *et al* (1988) further showed a distinct lymphokine secretion pattern for T cells obtained from immunized H-2^d, H-2^s and (H-2^d x H-2^s) F1 mice and interpreted these findings as the effect of Ir genes in the selective activation of Tinf and Th.

3) *In vivo* depletion of B cells by anti-μ antibodies resulted in defects in the priming of T cells that mediate DTH and *in vitro* proliferative responses (Herrmann *et al.*, 1988; Ron and Sprent, 1987), which could be due to a defect in the generation of TH1 as well as TH2 cells in B-cell-depleted mice.

With regard to the priming requirements for these cells, Janeway *et al* (1988) suggested that TH1 cells require higher concentrations of ligand than TH2, which can be achieved by B cells through efficient uptake of antigen via surface sIg. In contrast, TH2, which require IL-1 for their autocrine growth, do not require B cells for their priming, and are most likely primed by macrophages. Thus, the ligand density is the determining factor for the activation of TH1 and TH2 cells. Recently, Mosmann and Coffman (1989) proposed that the activation of TH1 cells lead to effector functions causing cytotoxicity which may be relevant in dealing with intracellular pathogens. TH2 activation, on the other hand, would induce the features of an allergic responses.

The biological properties of the lymphokines secreted by activated TH1 and TH2, with reference to their effect on APC and T cells are shown in Table 1.1.

8: Flaviviruses:

The Flaviviridae are a family of 65 currently recognized arthropod-borne viruses of medical importance. Amongst these, 28 have been associated with diseases in human beings, 3 of which, dengue, Japanese encepalitis (JE) and yellow fever, are of major public health importance (Monath, 1986).

The prototype flavivirus infection, yellow fever, is characterized by fever, hepatic, renal and myocardial dysfunction and haemorrhage. Despite the use of a live attenuated vaccine (17 D), 250,000 people in West Africa have been affected over last 20 years (Monath, 1986). This disease is also prevalent in tropical America.

Four sero-types of dengue virus are prevalent in endemic in the Carribean, Southeast Asia, and East Africa and Oceania. This disease is characterized by fever, rash, headache, and arthralgia. Severe complications of dengue virus infections are dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS). The major pathophysiological hallmarks include, haemorrhagic manifestations (vascular injury, thrombocytopaenia, and coagulopathy), pleural effusion, and shock which occur almost exclusively in persons with secondary infection with a heterologous serotype (Halstead *et al.*, 1970; Halstead,1989). This syndrome occurs annually in epidemic form in Southeast Asia, and occurred in Cuba for the first time after the second World War, in 1981 Despite many years of research

effort, vaccine development still remains at the experimental stage. Recently, the World Health Organization has targetted dengue viruses for intensive work toward vaccine development (Brandt, 1988).

JE occurs in endemic and epidemic form over a wide area of Asia including, the Indian-subcontinent, China, Nepal, Burma, Sri Lanka, Indo-China and Thialand (Monath, 1986). At least tens of thousands of cases, with a fatality rate of 20%, are reported annually. The disease has increased in importance during recent years and foci have been detected where it was previously rare or absent. Illness is manifested by fever, aseptic meningitis or encephalitis, vomition, dizziness and drowsiness. An inactivated adult mouse brain JE vaccine has been widely used in Japan. Although the technology for producing this vaccine has been transferred to other countries, the cost of production may make large scale production impracticable in certain Southeast Asian contries. In China, safety of a live attenuated JE vaccine produced in primary hamster kidney has been evaluated in children (Xin *et al.*, 1988). Although this vaccine gives a reasonable protection, the disease continues to occur with over 10,000 cases reported per year. Attenuated vaccines have been developed for pigs, a major reservoir species of this virus, and successfully used in China and Japan (Huang, 1982).

The mosquito-transmitted, West Nile virus (WNV) is widely distributed in different parts of the world including, Africa, Europe, USSR, Middle East and Asia. Virus isolates from different areas show antigenic variations probably due to frequent intercontinental exchange of this virus by migrating birds

(Monath, 1985). On clinical grounds West Nile fever resembles dengue (Monath, 1985).

Tick-borne encephalitis is clinically the most important European arbovirus infection. Infections with this virus have been reported from Austria, Czechoslovakia, Poland, FRG, Hungary, Switzerland, and USSR.

Kunjin virus is indigenous to eastern and northern Australia and is within the serological complex comprising West Nile, JE, St. Louis encephalitis (SLE) and Murray Valley encephalitis (MVE). This virus is widely distributed in Australia and often in association with MVE but it is rarely associated with clinical infection (Muller, 1986). Sporadic epidemic encephalitis due to MVE occurs in Australia, but its significance as a public health issue is limited.

Although many of these agents can be transmitted by aerosols or by body fluids, such as milk, urine or blood, in most of the cases a complex pattern of interactions involving virus, arthropod vector, vertebrate host, and the ecosystem, determine the intensity and the pattern of virus transmission. These viruses are capable of indefinite survival in insects by transovarial (from one generation to the next, in mosquitoes) and trans-stadial transmission (from one developmental stage to the next, in ticks). For arboviruses, with few exceptions, large vertebrates are typically amplifying hosts. Man can be implicated either as an incidental host in a dead-end cycle or as an active reservoir host causing other human infections, either by direct contact or through specific insect vectors. In tropical countries where the arthropod vector is plentiful year round, some of the diseases are endemic. In regions subjected to monsoonal rains, epidemics of mosquito-borne diseases may occur toward the end of the wet

season (e.g JE in Southeast Asia). Man-made changes in the ecosystem such as construction of dams, in irrigation projects in the arid regions have provided a breeding habitat for mosquitoes and water birds, resulting in the formation of enzootic foci in areas in which they were previously unknown. A different species of mosquito, with anthropophilic habits, may become involved in transmission of the virus in humans, and may then maintain the virus in man-mosquito-man, e.g., *Aedes aegypti*, the mosquito vector of dengue and urban yellow fever.

8.1. Classification and structure of flaviviruses:

All flaviviruses share a group-specific antigen (Brinton, 1986). On the basis of serological cross-reactivity and the type of arthropod vector involved in transmission during the natural cycle, flaviviruses are grouped into seven complexes (Table 1.2) (Brinton, 1986). The viruses discussed in this thesis belong to sub-group III which includes, JE, WN, SLE, MVE and Kunjin.

Flaviviruses contain a central nucleocapsid surrounded by an envelope. Virions are spherical and have a diameter of about 40-50 nm. Genomic single-stranded RNA is of positive polarity (plus-stranded) and is infectious (Brinton, 1986). The nucleotide sequence of several flaviviruses are now available, including yellow fever (Rice *et al.*, 1985), MVE (Dalgarno *et al.*, 1986), West Nile (Castle *et al.*, 1986), JE (Sumiyoshi *et al.*, 1986), dengue-4 (Mackow *et al.*, 1987), SLE (Trent *et al.*, 1987), and Kunjin (Coia *et al.*, 1988). All these studies indicated that the genome of flaviviruses consists of a single-stranded RNA of nearly 11kb, which encodes from 5' end the structural proteins, core (C), membrane (M) protein

precursor (prM), and envelope (E), followed by the non-structural proteins NS1, NS2A, NS2B, NS3, NS4A, and NS5.

Flaviviruses can replicate in a wide variety of cells of vertebrates and some arthropods. The initial latent phase of the flavivirus replication cycle lasts for 12 hours; after which progeny virus begins to be released from the infected cells. Maximal titres of virus do not begin to be produced until 24 hours after infection (Brinton, 1986). The synthesis of high levels of cell proteins continues throughout the flavivirus replication cycle (Brinton, 1986). The genomic 40S RNA is the initial template for minus strand complementary RNA, which subsequently becomes the template for the synthesis of progeny plus-stranded RNAs. These newly synthesized plus-stranded RNAs are utilized as templates for the production of more minus-strand RNAs, as mRNA, and as molecules for encapsidation into progeny virions. Translation is initiated close to the 5' end of the RNA and it is thought to result in a long peptide chain containing all the viral proteins (Coia *et al.*, 1988). Signal sequences precede the amino terminus of prM, E and NS1. Various structural and non-structural proteins usually detected in flavivirus-infected cells are shown in Table 1.3.

8.2. Immune response to flaviviruses:

A great body of information about immune mechanisms involved during pathogenesis of flaviviruses has been derived from experiments with mice and other laboratory rodents using adapted strains of viruses. The following is a brief literature survey on the immune response to flaviviruses.

In murine models, flaviviruses induce specific haemagglutination-inhibition and neutralizing antibodies of both IgM and IgG classes by 4 to 6 days after infection. IgM antibodies predominate in the early phase of infection and persist for up to 3 weeks; this is followed by the appearance of IgG antibodies. Neutralizing antibodies are primarily directed against the viral E and M proteins (Morath, 1986). The E glycoprotein binds to red blood cells and is probably involved in haemagglutination. Complement-dependent cytotoxic antibodies directed against membrane-associated non-structural protein, NS1, have also been shown to protect the mice from yellow fever (Schlesinger *et al.*, 1985, 1986) and dengue challenge (Zhang *et al.*, 1988). Antibodies directed against non-structural proteins on the surface of infected cells appear relatively late, but may play an important role in viral clearance (Morath, 1986). Antibody-dependent, complement-mediated cytotoxicity has been demonstrated *in vitro*, using Raji cells infected with dengue (Kurane *et al.*, 1984). Recent studies of antibody responses in human cases (Burke *et al.*, 1985) revealed that, 1) antibodies are not produced locally in CNS in inapparent infections, 2) fatal encephalitis is generally associated with either absence, lower or delayed levels of appearance of specific IgM and IgG antibodies in serum and cerebrospinal fluid, and, 3) patients recovered from encephalitis show high levels of serum and local CNS antibody responses.

With flaviviruses, the classical form of non-specific, NK-cell mediated cytotoxicity has been reported with splenocytes and PEC from mice on day 2 post-infection with Kunjin (MacFarlan *et al.*, 1977) and with peripheral blood leukocytes (PBL) in humans

from day 8 to 12 post-immunization with yellow fever (Fagaeus *et al.*, 1982). Homchampa *et al* (1988) assayed NK cell activity against K-562 target cells and HNK-1$^+$ cell levels (determined by immunofluorescence) in PBL of Thai children with dengue haemorrhagic fever/dengue shock syndrome, during febrile stage, shock (1-2 days of subsidence of fever), early convalescent (3-4 days of subsidence of fever) and late convalescent (14-18 days of subsidence of fever) stages. The study revealed that the levels of HNK-1$^+$ cells were significantly decreased in the febrile and shock stages and were normal in the early and late convalescent stages. However, on a per cell basis NK activity (determined as percentage cytotoxicity of HNK-1$^+$ cells for each sample) was significantly increased in the early stages of the disease with a peak during shock. These results indicated that NK cells may play some role in the pathogenesis of DHF/DSS.

With regard to T cell responses, various groups have studied DTH responses, T-cell cytotoxicity, and proliferative T cell responses. Most studies have shown the onset of such responses either simultaneously or shortly before the appearance of serum antibodies. Tc cell activity was demonstrated in Banzi virus-infected mice by day 6, was maximal by day 8, and decreased to background levels by day 16 (Sheets *et al.*, 1979). Gajdosova *et al* (1981) reported Tc cell responses against Langat, a member of the tick-borne encephalitis group, from 2 to 9 days post-infection, with a peak on day 6. Kesson *et al* (1987, 1988) studied the primary *in vivo* and secondary *in vitro* Tc cell responses to WNV. Primary WNV-immune T cells from CBA/H (H-2^k) mice showed K^k-restricted Tc cell activity from 4 to 7 days post-priming, with a peak on day 5. A potent secondary

WNV-immune Tc cell response *in vitro* was also studied to evaluate H-2 restriction patterns. These responses were mapped to the *K* region in the $H\text{-}2^k$ haplotype and both *K* and *D* regions of the $H\text{-}2^d$ haplotype. Pang *et al* (1988) have shown a dengue virus-2-specific *in vitro* secondary Tc cell response in CBA mice. Flaviviruses, Langat and yellow fever (Gajdosova *et al.*, 1980), dengue and JE (Pang *et al.*, 1988) and WN and Kunjin (Hill, 1990) are also known to cross-react at the level of Tc cell recognition.

Studies on MHC class II-restricted T cell reponses by measuring inflammatory infiltration after foot pad challenge have been reported in SLE (Hudson *et al.*, 1979), dengue (Pang *et al.*, 1982) and JE (Mathur *et al.*, 1983). These studies revealed that peak responses were observed on day 6 after priming. In a murine model, Rothman *et al* (1989) demonstrated that dengue-immune L3T4$^+$ T cells obtained from dengue 4 virus-primed BALB/c mice, predominantly responded to preparations containing a combination of C, pr-M, E, NS1 and NS2a proteins of dengue 4 virus, expressed in Sf9 cells with a recombinant baculovirus, and to a lesser extent, to the dengue 4 virus E protein alone. Cross-reactivity at the level of Th cell recognition in WNV, Kunjin and MVE viruses has been reported by Uren *et al* (1987).

The broad context for evaluation of the role of T cell responses in flavivirus infections is as follows.

Firstly, the importance of T cells in recovery from acute viral infections has been well established (Blanden, 1974). Acute and persistent lymphocytic choriomeningitis virus (LCMV) infection can also be cleared by T cells (Mims and Blanden, 1972; Oldstone *et al.*, 1986) even from the substance of the brain, without direct

vascular access (Oldstone *et al.*, 1986). However, in the LCMV-infected CNS, T cell-mediated immune mechanisms can contribute towards immunopathology, (Cole *et al.*, 1972; Zinkernagel and Doherty, 1973). It may be that such mechanisms are involved in the pathogenesis of encephalitis produced by flaviviruses.

Secondly, large numbers of flaviviruses co-circulate in areas of endemic human disease. Serological cross-reactions amongst flaviviruses have been reported both *in vitro* (Westway, 1965; Madrid and Porterfield, 1974) and *in vivo* (Price, 1963; Henderson *et al.*, 1970; Kayser *et al.*, 1985) on the basis of cross-protection studies. Recent work suggests that MAbs directed against several epitopes mapped on glycoprotein E cross-react extensively with other flaviviruses (reviewed by Roehrig, 1986). Cross-protection from challenges with SLE, JE and MVE (Roehrig, 1983) using non-neutralizing, cross-reactive MAbs has been reported. However, such cross-reactive antibodies also enhance the replication of certain flaviviruses including dengue and yellow fever, in Fc receptor-bearing cells (Halstead and O'Rourke, 1977, Peiris and Porterfield, 1979, Halstead *et al.*, 1980; Gould *et al.*, 1987).

Cross-reactivity amongst flaviviruses is of particular importance in the pathogenesis of DHF/DSS. These syndromes occur almost exclusively in persons who experience a secondary infection with a heterologous dengue serotype (Halstead *et al.*, 1970), indicating the probable association of these syndromes with the immune status of an individual. This was shown to be probably due to complexes, formed by pre-existing, non-neutralizing, heterotypic IgG antibodies with virus, which promote both attachment to and infection of Fc-bearing monocytes (Halstead and O'Rourke, 1977;

Halstead *et al.*, 1980; Gollins and Porterfield., 1984). Evidence for this was derived largely from *in vitro* studies employing PBL and macrophage cell lines. The results obtained from experimental sequential infections of monkeys (Halstead *et al.*, 1973) and challenge after passive administration of antibodies (Halstead *et al.*, 1979) support this concept. In these monkeys, levels of viraemia and virus titres in tissues were higher than in non-immune control monkeys. Anamnestic antibody responses early in the course of secondary infection are typically cross-reactive. Other possible mechanisms involved in the pathogenesis of DSS include, complement activation, histamine release and consumptive coagulopathy (reviewed by Halstead, 1989).

In addition, the role of T cells in the pathogenesis of DHF/DSS has also been postulated (Kontny *et al.*, 1988). Proliferation and production of IFN-γ was shown in PBL cultures derived from dengue-immune donors when stimulated with dengue 3 virus (Kurane *et al.*, 1989). Since this lymphokine is known to increase Fcγ R (Guyre *et al.*, 1983) on monocytes which support dengue virus replication, these authors suggested that IFN-γ-producing CD4+ T cells may augment dengue virus infection of human monocytes. In addition, Kontny *et al* (1988) showed that IFN-γ acts synergistically with non-neutralizing antibodies in the enhancement of infection in monocytes. It is likely that cross-reactivity at the level of Tc and Th cell recognition of flavivirus antigens may contribute toward the immunopathology associated with DHF and DSS, probably involving production of IFN-γ (recent evidence suggests that Tc cells also secrete IFN-γ) (Mosmann & Coffman, 1989). It is also likely that IL-2 and IFN-γ, produced by inflammatory T

cells will augment the production of tumour necrosis factor α and β in monocytes (Nedwin *et al.*, 1985). Swelling and hyaline necrosis of Kupffer cells, commonly observed in histologic examination (reviewed by Halstead, 1989) may be due to tumour necrosis factors secreted by activated monocytes. Taken together, these findings strongly suggest the possible role of the products of activated monocytes and inflammatory T cells in the pathogenesis of DSS.

Understanding of the nature of viral antigens involved in recovery and the protective immunity on one hand and immunopathology on the other hand will provide insight into the rational development of future vaccines. In the case of arboviruses, with increasing difficulty in the control of vectors because of acquired resistance to pesticides and inaccessibility of certain vectors to control measures, vaccines would provide an important approach to reduce the incidence of such infections in the populations at risk. Studies described in this thesis represent a step towards the identification of sources of flavivirus epitopes recognized by $CD4^+$ T cells in mice. Although the murine models do not directly contribute towards human vaccine development or understanding of disease processes in humans, detailed investigation of cellular immune responses in laboratory animals in the absence of intercurrent infections and under defined conditions *in vitro* can provide fundamental information which is complementary to clinical studies in humans.

Table 1.1* : Biological effects of lymphokines secreted by activated T cells on B cells, macrophages and T cells.

Lymphokine	Synonyms	Sources	Biological activities.
IL-2	T cell growth factor (TCGF), T cell maturation/ stimulating factor (TMF/TSF), Killer helper factor (KHF), T cell replacing factor (TRF).	Activated TH1 cells, Tc cells.	T cells:- Induction of proliferation of resting T cells, growth of activated TH1 & TH2 cells, Tc cell generation. B cells:- Proliferation of B cells with anti-Ig + lipopolysaccharide (LPS), proliferation and differentiation of antigen-activated B cells, induces plaque forming cell responses to SRBC in the presence of IL-1 and IFN-γ from B cells, induces IgM secretion from B cells.
IL-4	B cell growth factor-1 (BCGF-1), B cell stimulatory factor-1 (BSF-1), T cell growth factor II (TCGF-II), Macrophage activating factor (MAF).	Activated TH2 cells.	T cells:- Proliferation of resting T cells with phorbol myristate acetate (PMA), increased IL-2-receptor (IL-2R) expression in the presence of PMA, growth of activated TH1 & TH2 cells. B cells:- Induction of class II MHC molecules on resting B cells, induction of FcεR on resting B cells, increased secretion of IgG1 & IgE by LPS-activated B cells or B cells stimulated with T cells & antigen; down regulation of IgG2a, IgG2b & IgG3 & IgM secretion by LPS-activated B cells. Increased class II MHC expression on macrophages.

Table 1.1 contd.

IL-5	B cell growth factor II (BCGF-II), T cell replacing factor (TRF), killer helper factor (KHF), eosinophil-activating factor (EAF)	Activated TH2 cells	T cells:- Induces generation of Tc cells from peanut agglutinin-positive thymocytes in presence of IL-2. B cells:- Increased IL-2-R expression on activated B cells, increased IgM & IgG secretion, enhanced IgG1 & IgE secretion with IL-4, increased IgA secretion by LPS-activated B cells.
IL-6	Plasmacytoma growth factor, B cell stimulatory factor-2 (BSF-2) (human), IFN-β2 (human)	Activated T cells, Macrophages.	T cells:- Induces Tc cell activity with IL-2 & IFN-γ, co-factor for the expression of IL-2 R (human T cells) B cells:- Proliferation & Ig secretion by activated normal B cells.
IFN-γ	Macrophage activating factor	Activated TH1 cells & Tc cells	T cells:- Inhibition of IL-4-induced proliferation of TH2 but not TH1 cells. Induction of IgG2a production in the presence of LPS, decreased IL-4-induced activities, differentiation of B cells, increased MHC class I & class II expression on many cells, enhancement of tumour necrosis factor, lymphotoxin production by macrophages, increased FcR expression.

* Modified from O'Garra *et al* (1988) and Vitetta *et al* (1989).

Table 1.2 : Antigenic and vector classification of flaviviruses. [1]

Mosquito-borne	Antigenic group[a]	Tick-borne	Antigenic group[a]	Vector unknown	Antigenic group[a]
Alfuy	III	Kadam	I	Carey Island	I
Bussuquara	III	karshi	I	Negishi	I
Japanese encephalitis (JE)	III	Kyasanur Forest disease	I	Phnom Penh bat	I
Kunjin	III	Langat	I	Cowbone Ridge	II
Murray Valley encephalitis (MVE)	III	Louping ill	I	Jutiapa	II
Saint Louis encephalitis (SLE)	III	Omsk hemorrhagic fever	I	Moc	II
Stratford	III	Powassan	I	Sal Vieja	II
Usutu	III	Royal Farm	I	Erlita	II
West Nile (WN)	III	Saumarez Reef	I	Koutango	III
Bagaza	IV	Tick-borne encephalitis (TBE)	I	Israel turkey meningo-encephalitis	VII
Kokobera	IV	Russian spring-summer	I	Apoi	VII
Ntaya	IV	Tyuleniy	U	Bukalasae bat	VII
Tembusu	IV			Dakar bat	VII
Yokose	IV			Entebbe bat	VII
Banzi	V			Rio Bravo	VII
Bouboui	V			Saboya	VII
Edge hill	V			Aroa	U
Uganda S	V			Cacipacore	U
Dengue-1	VI			Gadgets Gully	U
Dengue-2	VI			Montana myotis leuko-encephalitis	U
Dengue-3	VI			Rocio	U
Dengue-4	VI			Sokuluk	U
Ilheus	U				
Jugra	U				
Naranjal	U				
Sepik	U				
Sponweni	U				
Yellow fever (YF)	U				
Wesselsbron	U				
Zika	U				

[a] Antigenic groups are designated I, II, III, IV, V, VI, VII, and U (unknown)

[1] Reproduced from Brinton, (1986).

Table 1.3 : Kunjin-virus specified proteins.

Protein	Calculated size *	Properties
Structural Proteins		
C	13.4	nucleocapsid protein
prM	18.4	precursor to minor virion envelope protein M
E	53.7	major virion envelope protein
Non-structural Proteins		
NS1	39.8	soluble complement fixing antigen
NS2A	25.4	hydrophobic, function unknown
NS2B	25.4	hydrophobic, function unknown
NS3	68.9	RNA polymerase, protease?
NS4A	16.1	hydrophobic, function unknown
NS4B	27.5	hydrophobic, function unknown
NS5	103.6	replicase component?

* Adapted from Coia *et al* (1988).

CHAPTER 2

IN-VITRO T CELL PROLIFERATIVE RESPONSE TO THE FLAVIVIRUS, WEST NILE

Introduction

Currently twenty-eight different flaviviruses are known to cause infections in humans in many countries throughout the world (Monath, 1986). The clinical manifestations associated with these infections include; an acute encephalitis, e.g. JE, MVE or SLE; fever, rash and arthralgia, e.g. West Nile fever or dengue fever; and haemorrhagic fever, e.g. yellow fever, dengue fever and shock syndrome. There is evidence to substantiate the importance of humoral antibodies in virus neutralization (Phillpotts and Porterfield, 1985; Gollins and Porterfield, 1986) and in immunopathology in dengue shock syndrome (reviewed by Halstead, 1989). On the other hand, despite several reports on the ability of flaviviruses to induce DTH (Pang *et al.*, 1982; Allen and Doherty, 1986) and Tc cell responses (Kesson *et al.*, 1987; Pang *et al.*, 1988), little is known about the viral antigens recognized by Th cells (Rothman *et al.*, 1989) and the possible role of Th cell responses in recovery/resistance or pathogenesis of diseases. Studies with $CD4^+$ T cells from dengue-immune donors indicated that these cells produce IFN-γ and may contribute to the pathogenesis of dengue haemorrhagic fever and dengue shock syndrome (Kurane *et al.*, 1989).

Antigen recognition by Th cells is restricted by class II MHC gene products present on APC. The most extensively studied APC are DC, macrophages and B cells. These cells provide epitopes in the form of complexes between a self class II MHC molecule and a peptide derived from a foreign protein, which is recognized by specific Th cell receptors. Th cells, which

predominantly possess CD4+ (L3T4+) cell surface antigens (Swain, 1983) respond during infection and provide help for antiviral antibody production through the production of IL-2, IL-4 and IL-5 (Snapper and Paul, 1987a,b; Swain *et al.*, 1988; Poo *et al.*, 1988). Recently, antigen-activated Th cells have also been shown to produce IL-6 which is involved in the differentiation of Tc cells (Okada *et al.*, 1988).

A great body of information about flavivirus pathogenesis has been derived from experiments with mice and other laboratory rodents using adapted strains of viruses. West Nile virus (WNV) is a mouse-infectious member of the antigenic complex of flaviviruses which include MVE, SLE and JE. This Chapter describes the investigation of various parameters of murine Th cell proliferative responses against WNV-encoded antigens using enriched populations of memory CD4+ T cells as responders *in vitro* and semi-defined Ia+ APC from splenocyte populations. This represents the first step towards answering fundamental questions such as, which viral gene products give rise to Th cell epitopes, the nature of Ir gene control of Th responses and the efficiency of various defined classes of APC.

Materials and Methods

Mice:

Inbred CBA/H (H-2^k) mice, bred at the John Curtin School of Medical Research (JCSMR) under specific pathogen-free conditions were used at 8 to 10 weeks of age.

Viruses:

WNV, Sarafend strain was grown in 4 day-old suckling mouse brain (Kesson *et al.*, 1988) and titrated by standard plaque assay (Taylor and Marshall, 1975). This stock virus was designated as WNV-B.

Uninfected 5 day-old CBA/H brain suspension was prepared similarly and used in mock infection.

WNV-B was passaged five times in Vero cells and a second virus stock (WNV-V) was prepared from the cell lysates of the fifth passage and titrated as described above. When necessary, WNV-V stock virus was UV-irradiated in 2 ml aliquots for 10 min at 1.09 W/cm^2.

Influenza A/WSN stock was prepared by standard methods (Yap and Ada, 1977).

Immunization of mice:

Mice were normally injected intravenously (i.v.) with 10^6 plaque forming units (pfu) of WNV-B, diluted in 200 µl of gelatin saline (0.5% gelatin in borate-buffered CaMg saline, pH 7.2) at 4^0 C. In experiments designed to optimize the dose and route of priming, virus doses of 10^2 to 10^8 pfu were inoculated i.v., intraperitoneally (i.p.) or s.c. in the flank. A dose of 10^3 HAU of influenza A/WSN diluted in 200 µl gelatin saline was used to prime mice i.v. as a specificity control.

Vero cell-derived WNV antigen:

Monolayers of low passage (passage no. 45) Vero cells in 80 cm^2 flasks (Nunclon, Denmark) were infected for 1 h at 37^0 C in a humidified atmosphere of 5% CO_2 in air, with WNV-V at a multiplicity of 100 pfu per cell using 2 ml of Eagle's minimal essential medium (EMEM, Gibco Laboratories, Grand Island, NY, USA) per flask.

Uninfected control cultures were incubated with 2 ml of plain EMEM. After 1 h, unadsorbed virus was removed by 2 washings with EMEM and the monolayers were cultured at 37^0 C in EMEM supplemented with 2% foetal calf serum (FCS), 200 U/ml of Penicillin G, 200 µg/ml Streptomycin and 125 µg/ml Neomycin. At 3, 6, 9 and 12 h post-infection, monolayers were washed twice with EMEM, 5 ml of EMEM was added to each flask and the flasks were stored at -70^0 C. Freeze-thawed cell lysates were then sonicated for 15 seconds using a Branson B12 sonifier (Branson Sonic Power Co., Conn, USA) at 50 W, followed by centrifugation at 7000 rpm at 4^0 C for 20 min. Two ml aliquots of supernatant were UV-irradiated for 10 min at 1.09 W/cm^2. Control cell monolayers were processed similarly.

EL-4 supernatant:

An IL-2 secreting EL-4 cell line (clone 23) was a gift of Dr. C.J. Sanderson, Mill Hill, London. EL-4 supernatant was prepared as described by Farrer *et al* (1980). Briefly, cells were cultured at 10^6/ml in RPMI 1640 (Gibco Laboratories, Grand Island, NY, USA) supplemented with 5% FCS and 10 ng/ml of phorbol myristate acetate (Sigma, St. Louis, MO., USA) for 24 h. The culture supernatants were then harvested after centrifugation to deposit the cells, sterilized by filtration (0.22 µm) and stored at -20^0 C.

Monoclonal antibodies:

Anti-Thy-1.2 (clone F7D5) was obtained from Serotec (Kidlington, UK). Hybridoma lines secreting anti-Lyt-2 (31m), anti-$L3T4^+$ (172) and anti-dendritic (33D1) were gifts from Dr.R.Ceredig and Dr.J.Allan. These cells lines were grown in DMEM (Dulbecco's Modified Minimum Essential Medium, Gibco Laboratories, Grand Island, NY, USA), supplemented with 5% FCS. The optimum

dilutions of anti-L3T4$^+$ and anti-Lyt-2$^+$ antibodies were determined as described by Ceredig *et al* (1985). The optimal dilutions of anti-dendritic antibodies required was determined by trypan blue dye exclusion test performed on purified splenic dendritic cells (Pereira *et al.*, 1986), treated with specific antibody and a 1/10 dilution of rabbit complement (Low Tox, Cedarlane, Canada).

Spleen cells:

Cell suspensions were prepared as described by Kesson *et al* (1988) and used as responder populations or partly purified to obtain enriched populations of responders and APC.

Enrichment of L3T4$^+$ T cell responder populations:

After initial adherence of splenocytes to a plastic surface for 2 h at 37°C, non-adherent cells were carefully removed without any agitation and treated with anti-Lyt-2 and anti-dendritic MAbs at 4^0 C for 30 min. Subsequently, these cells were rosetted with SRBC coupled with sheep anti-mouse IgG to remove all Ig$^+$ cells including Lyt-2$^+$ cells, B cells, dendritic cells and any remaining macrophages (via Fc receptor binding) (Parish and McKenzie, 1978). Non-rosetting cells were then separated on Isopac-Ficoll gradients and washed three times with DMEM. The responder populations enriched by this method were tested by trypan blue dye exclusion after treatment with either anti-Lyt-2 or anti-L3T4 antibodies and complement. Anti-Lyt-2-treated cells showed less than 3% dead cells, whereas treatment with anti-L3T4 antibodies showed 98% dead cells in trypan blue dye exclusion test. These populations hereafter will be referred to as CD4$^+$ T cells.

Enrichment of Ia+ APC:

Overnight incubation of splenocytes at 37°C on a plastic surface facilitated the separation of splenic macrophages (adherent) from dendritic cells (Steinman *et al.*, 1979) and lymphocytes (non-adherent). The non-adherent population was subsequently treated with anti-Thy-1.2 antibody and complement to kill T cells. The remaining viable cell populations separated on Isopac-Ficoll gradients contained B cells and SDC, both known to be Ia+ APC (Chesnut *et al.*, 1982; Inaba and Steinman, 1985).

Proliferation assay:

Responder splenocytes from WNV-primed mice, either whole populations or enriched for CD4+ T cells, were dispensed in triplicate in 100 µl DMEM supplemented with 5% FCS, antibiotics and $5x10^{-4}$ M 2-mercaptoethanol (2-ME), containing $2x10^5$ cells, into 96 well round-bottomed microtitre plates (Linbro, Flow Laboratories Inc., VA, USA). Three different types of stimulator populations: whole splenocytes from naive and primed mice, and enriched Ia+ APC from splenocytes of primed mice were used. These cells were either, 1) pulsed with 100 pfu/cell of WNV for 3 h; washed twice with DMEM and γ-irradiated (with 2000 rads from a ^{60}Co source) and then cultured with responders; or 2) γ-irradiated first and then cultured with responders in the presence of UV-irradiated WNV stock or a lysate prepared from WNV-V-infected Vero cells (in 50 µl vol). Stimulators and responders were co-cultured at a 1:1 ratio in a total vol. of 200 µl unless otherwise stated. After 48 h of incubation in a humidified atmosphere of 5% CO_2 in air at 37^0 C, 0.5 µCi/well (^3H) thymidine (in 50 µl aliquot) was added and cultures were harvested after 72 h onto filter paper using a Titertek (Flow Lab, UK) cell

harvester and counted in a liquid scintillation counter (Beckman, LS3801, USA).

Modified assay:

This assay was performed as described by Chain *et al* (1987). Briefly, $2x10^5$ responders and irradiated $2x10^5$ stimulators, each in 50 µl DMEM supplemented with 1% normal mouse serum (NMS) were incubated with 50 µl of WNV antigen (WNV-V-infected Vero cell lysate) for 72 h. After this, 75 µl of DMEM supplemented with 5% FCS and 8% EL-4 supernatant was added. Thymidine incorporation and harvesting were performed at 96 and 120 h respectively.

Responses of enriched responder populations to mitogens:

The presence of contaminating B cells in the responder populations was studied functionally by evaluating the responses to mitogens. Whole splenocytes or non-adherent populations prior to and after Lyt-2^+ and sIg^+ cell depletion were assayed for their response to Con A and bacterial lipopolysaccharide (LPS). These cells were dispensed at $2x10^5$ cells per well in 100 µl aliquots in 96 well round-bottomed microtitre plates in DMEM supplemented with 5% FCS. Con A and LPS were added independently at a final concentration of 5 µg and 100 µg/ml respectively, in 20 µl aliquots. Thymidine was added after 48 h and the cultures were harvested at 72 h as described above.

The responses to Con A were 5-fold lower in the enriched responder population than the whole spleen, indicating depletion of macrophages which are needed for optimal T cell responses with Con A (Mills *et al.*, 1976). The responses to LPS were 70-fold lower, indicating depletion of B cells. These observations

indicated that the responder populations were markedly depleted of contaminating cells which could act as APC.

Phenotype of stimulator populations:

APC populations used in the experiments described in Tables 2.3 and 2.4 and Figs. 2.2 and 2.3, were examined by flow cytofluorometry for the expression of sIg and Thy-1.2 surface markers, prior to and after the enrichment procedures. For Thy-1.2 staining, 10^6 viable cells were treated with 100 µl of biotin-conjugated anti-mouse Thy-1.2 antibodies (Becton Dickinson, USA) at a 1/100 dilution for 30 min at 4^0 C. Cells were then centrifuged in 12x75 mm polystyrene round-bottomed tube through a bed of 0.5 ml heat-inactivated bovine serum (BS) at 250g for 5 min, followed by staining with 100 µl of streptavidine R phycoerythrine conjugate (QB AR 4, Serotec, USA, 1/50 dilution) for 30 min at 4^0 C. Then the cells were washed again through a bed of BS. After one additional washing the cells were examined by quantitative cytofluorometry on a FACS IV model analyzer (Becton Dickinson, USA), 10,000 gated events were collected. Control cells were stained only with 100 µl of streptavidine R phycoerythrine conjugate. Staining for sIg expressing cells was done by using fluorescein-conjugated goat anti-mouse IgG antibodies (Silenius, Australia) at a 1/20 dilution). Non-specific binding of fluorescein conjugate was minimized by prior treatment of the cells with normal goat IgG (Silenius, Australia) at a 1/20 dilution. Control cells obtained from normal splenocytes depleted of Ig^+ cells (Parish and McKenzie, 1978), were stained similarly.

Statistical analysis:

Data are expressed as mean ^{3}H-TdR uptake (counts per minute) of at least triplicate cultures ± standard error of the mean (S.E.M), where appropriate, using Student's 't' test.

Results

Initial experiments were designed to determine the optimal conditions necessary for priming mice and *in vitro* restimulation of proliferation of their splenic T cells. Whole splenocyte populations from 14 day-WNV-primed mice were used as responder cell populations and WNV-B-pulsed splenocytes from naive mice were used as stimulators. T cell responses were measured in a 3 day proliferation assay. In these experiments it was observed that priming i.v. with 10^6 pfu, followed by *in vitro* restimulation with the stimulators pulsed with 100 pfu of WNV-B gave optimal responses.

Route of priming and kinetics of generation of primed T cells in mice:

Preliminary studies showed that proliferative T cell activity could not be detected in the first six days after priming. Doses of 10^6 pfu of WNV-B were inoculated i.v., i.p., and s.c. in the flank region; and splenocytes from such primed mice 7 to 21 days post-infection were tested for proliferation in 72 h cultures. Proliferative responses were detected on day 7, reached a peak on days 12 to 15 and declined thereafter by day 21. Similar kinetics of T cell activity were observed following inoculation by all routes tested, however mice primed i.v. consistently showed higher responses.

Kinetics of *in vitro* restimulation of WNV-primed splenocytes:

The optimum culture time for the generation of T cell proliferative responses was studied using splenocytes from 14-day primed mice. The proliferative responses were measured at 12, 24, 48, 60, 72 and 96 h after the establishment of cultures. Responses were maximal at 72 h and declined slightly at 96 h (Fig. 2.1).

Antigenic requirements for *in vitro* restimulation:

The optimal conditions established thus far were used to evaluate various antigen preparations for restimulation of primed T cells. Responder splenocytes from mice primed i.v. 10, 15 and 21 days previously with 10^6 pfu WNV-B were cultured at a 1:1 ratio with splenocytes from naive mice pulsed with infectious, or an equivalent concentration of UV-irradiated preparations, of WNV-B or WNV-V.

Infectious WNV-B elicited proliferative responses from the splenocytes of all primed mice, but WNV-V suppression of all responses (Table 2.1). UV-irradiated WNV-B or WNV-V stocks stimulated comparable responses from all primed mice (Table 2.2). These results raised the possibility that infectious WNV may infect and kill T cells unless the latter are protected by mouse interferon present in the virus stock (as in WNV-B, not WNV-V). Therefore, the efficacy of antigen preparations containing virus-specified proteins but with a minimum of infectious WNV was investigated.

Restimulation of primed splenocytes with lysates of WNV-V-infected Vero cells:

Lysates prepared from WNV-V-infected Vero cells at 3, 6, 9 and 12 h post-infection were used to pulse APC at various concentrations. In order to minimize the background proliferation due to non-CD4+ cells, responder splenocytes from 14 day WNV-B-primed mice were enriched for CD4+ T cells as described in Materials and Methods (Pp. 60). Since antigen-specific B cells have been shown to present antigen efficiently (Lanzavecchia, 1985), APC from the splenocytes of mice primed with WNV 14 days previously were used and enriched for B and dendritic cells as described in Materials and Methods (Pp. 61).

Vero cell lysates showed increasing antigenicity with increasing time after infection; thus only 9 or 12 h lysates gave significant stimulation, with the latter being better (Fig. 2.2). Also, undiluted lysates suppressed T cell proliferation, whereas 1/4 or 1/8 dilutions were stimulatory. Further experiments used 1/4 diluted 12 h lysate. The assay used in this experiment was a 5 day modified protocol (see Materials and Methods Pp. 62) which is evaluated in a later section.

Virus specificity of T cell proliferation:

The specificity of T cell responses against WNV-infected 12 h Vero cell lysate was tested by comparing the proliferative responses of CD4+ T cells from 14 day WNV-primed, influenza-primed or naive mice using APC from 14 day WNV-primed mice. As shown in Table 2.3, responses obtained from WNV-primed responders were more than 10-fold higher than those from influenza-primed or naive responders. The influenza-primed

$CD4^+$ control T cells proliferated strongly in the presence of influenza antigen (Table 2.3).

Analysis of stimulator to responder ratios:

Using $CD4^+$ enriched WNV-primed responder populations, APC from WNV-primed mice and 12 h lysates from WNV-infected Vero cells (1/4 dilution), various ratios of stimulators to responders (0.1 to 10) were used to test effects on T cell proliferation. Optimal responses were obtained at a stimulator to responder ratio of 1 (Fig. 2.3). High numbers of stimulator cells suppressed T cell proliferation.

Modification of the T cell proliferation assay:

Changes in the medium composition and time of cultures were made to investigate effects on specific responses and backgrounds. In 3 day cultures a higher degree of antigen-specificity was observed with 1% NMS than with 5% FCS (Table 2.4). In order to amplify antigen-specific proliferation, the assay was further modified by adding an exogenous source of IL-2 (in the form of EL-4 supernatant) on the third day of a 5 day culture. This modification (using 1% NMS) significantly increased antigen-specific proliferation while not significantly increasing the background.

Phenotype of APC populations:

The distribution of fluorescence intensity of the APC populations prior to and after the enrichment procedures are shown in Fig. 2.4 A, B and Fig. 2.4.C, D respectively. The percentage of Ig^+ cells was increased by 20% after the enrichment (Fig. 2.4 A and C), whereas Thy-1.2^+ cells were almost absent in the enriched population (Fig. 2.4 B and D). In light of the protocol used (see Materials and Methods pp. 61), it is possible that these populations also

contained a proportion of SDC, which are normally present in very small numbers (1 % or less of the total cell population).

Discussion

This Chapter describes investigations of fundamental variables relevant to Th cell responses against WNV-encoded antigens. Preliminary experiments investigated doses of WNV required to prime Th cells for secondary proliferative responses *in vitro* using splenocytes from primed animals as responder cells and unprimed splenocytes as a source of antigen presenting cells. These experiments revealed that low doses of 10^2 or 10^4 pfu of WNV i.v. were inadequate but doses of 10^6 were satisfactory, whereas higher doses up to 10^7 or 10^8 pfu killed mice. The i.v. route of priming was found to be superior to i.p. or s.c. routes. At least 7 days were needed after i.v. priming before memory T cell populations capable of good proliferative responses *in vitro* were present in the spleen. Thereafter, memory was maintained until at least 21 days and presumably persisted longer.

Various kinds of antigen preparations were tested for efficacy in pulsing normal splenocytes as a source of antigen presenting cells. For this preliminary work, responder Th cells were obtained from 14 day-primed splenocyte populations following i.v. priming with 10^6 pfu of WNV. Thymidine incorporation was assessed after 3 days of culture in medium containing 5% FCS. Under these experimental conditions, background proliferation was often present but antigen-specific proliferation was sufficiently higher so that different antigen sources could be compared. In this preliminary system, WNV

stocks grown in mouse brain were a satisfactory source of antigen, either in infectious form or after UV irradiation to inactivate virus. In contrast, WNV stocks grown in Vero cells were satisfactory only if they were UV-irradiated. This suggested that infectious WNV had an adverse effect on Th cell proliferation and that in the case of mouse brain stocks, interferon in the crude stock protected the Th cells.

In order to avoid the problem of infectious virus preventing Th cell proliferation, lysates of WNV-infected Vero cells prepared at short intervals after infection, before production of progeny virions, were tested. Lysates were prepared at 3, 6, 9 and 12 h after infection. 12 h Vero cell lysates were superior to lysates harvested at earlier time intervals, presumably because insufficient viral protein had been synthesized at the earlier times. Using 12 h lysates and enriched $CD4^+$ responder T cells from 14-day primed spleen it was shown that a mixture of B cells and SDC operated as APC in this system.

In order to reduce the background proliferation characteristic of proliferative responses in media containing FCS, a modified assay was developed, in which an initial exposure of primed Th cells to APC pulsed with 12 hour Vero cell lysates was conducted for 3 days in medium containing 1% NMS, followed by a further 2 day culture period in medium containing EL-4 supernatant as an exogenous source of IL-2 as well as a variety of other T cell growth-promoting factors. This bi-phasic system reduced background proliferation but allowed very strong antigen-specific proliferation.

These results will allow future investigation in much more detail of essential parameters regulating Th cell responses during

flavivirus infection. These parameters include the efficacy of various different APC such as B cells, macrophages and SDC, Ir gene effects of different MHC class II genes and the nature of the flavivirus gene products which best stimulate Th cell responses. Information from such studies will aid better understanding of the pathogenesis of flavivirus infection and a more rational basis for protective vaccines.

Summary

WNV-specific murine T cell proliferation *in vitro* was investigated in terms of parameters which optimise antigen-specific responses and reduce background proliferation. The responder populations comprised splenocytes from WNV-primed mice, enriched for $CD4^+$ T cells. Ia^+ APC were derived from splenocytes of WNV primed or naive mice. Antigen was a lysate prepared from WNV-infected Vero cells at 12 h post-infection. Strong virus-specific proliferative responses were observed when antigen-pulsed APC were co-cultured with responders at a 1:1 ratio. Substantial non-specific proliferation occurred when FCS-supplemented culture medium (5% serum) was used, whereas with 1% NMS, a higher degree of antigen-specificity was evident, although the magnitude of responses was lower. The best separation between antigen-specific and background proliferation was obtained by using an exogenous source of T cell growth factors to amplify for 2 days the proliferation of $CD4^+$ T cells triggered by an initial 3 days of culture with antigen-pulsed APC. This investigation has defined optimal conditions for

investigating the stimulation of WNV-primed CD4+ T cell proliferation in response to the presentation of viral gene products by Ia+ APC. This assay should permit detailed analysis of the efficiency of various APC populations and identification of viral antigens that stimulate the proliferation of class II MHC-restricted T cells.

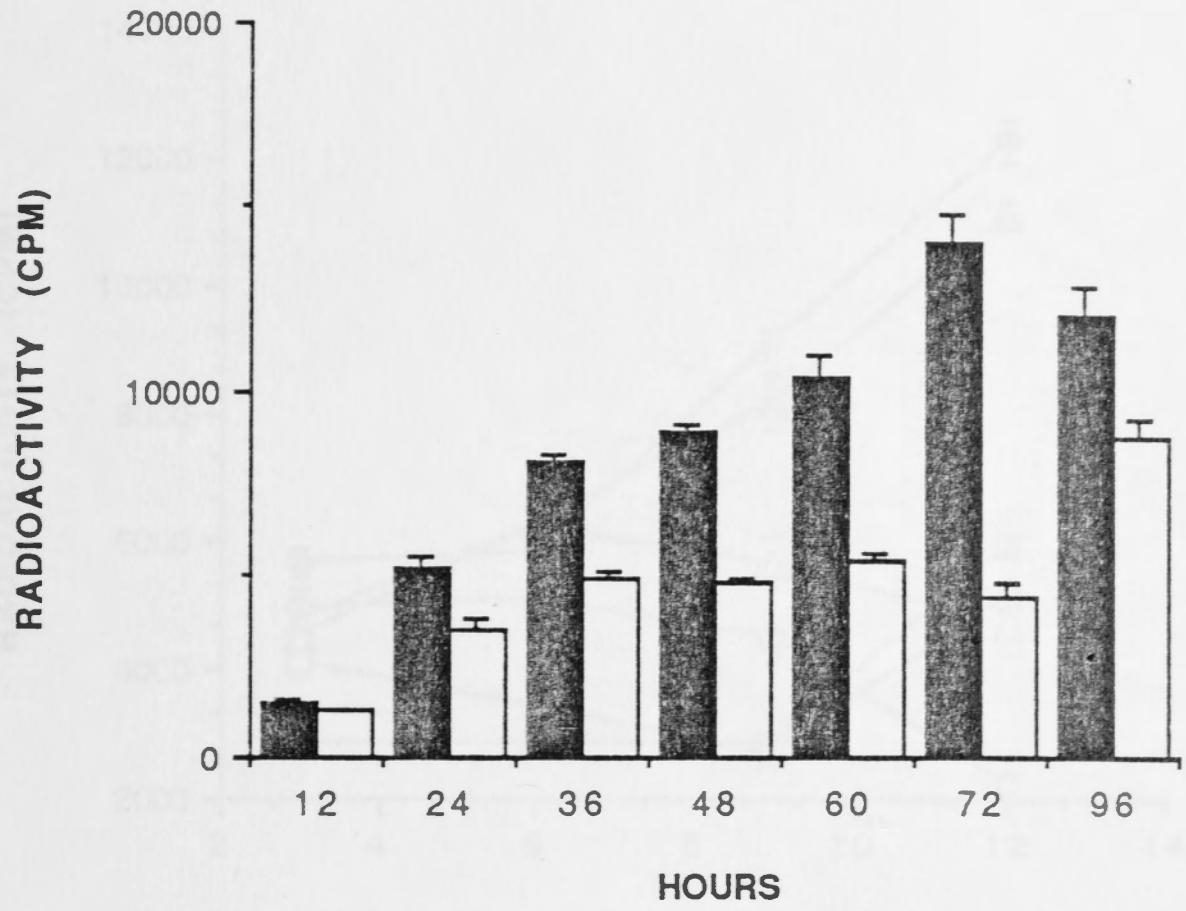


Fig. 2.1. Kinetics of *in vitro* restimulation of WNV-primed splenocytes. Splenocytes from 14-day primed mice were cultured at a 1:1 ratio with irradiated stimulators, treated either with WNV-B at 100 pfu/cell (■) or with control mouse brain antigen (□). ^{3}H-TdR was added to the cultures 12 h before termination. Results represent the mean ^{3}H-TdR uptake by triplicate cultures ± S.E.M.

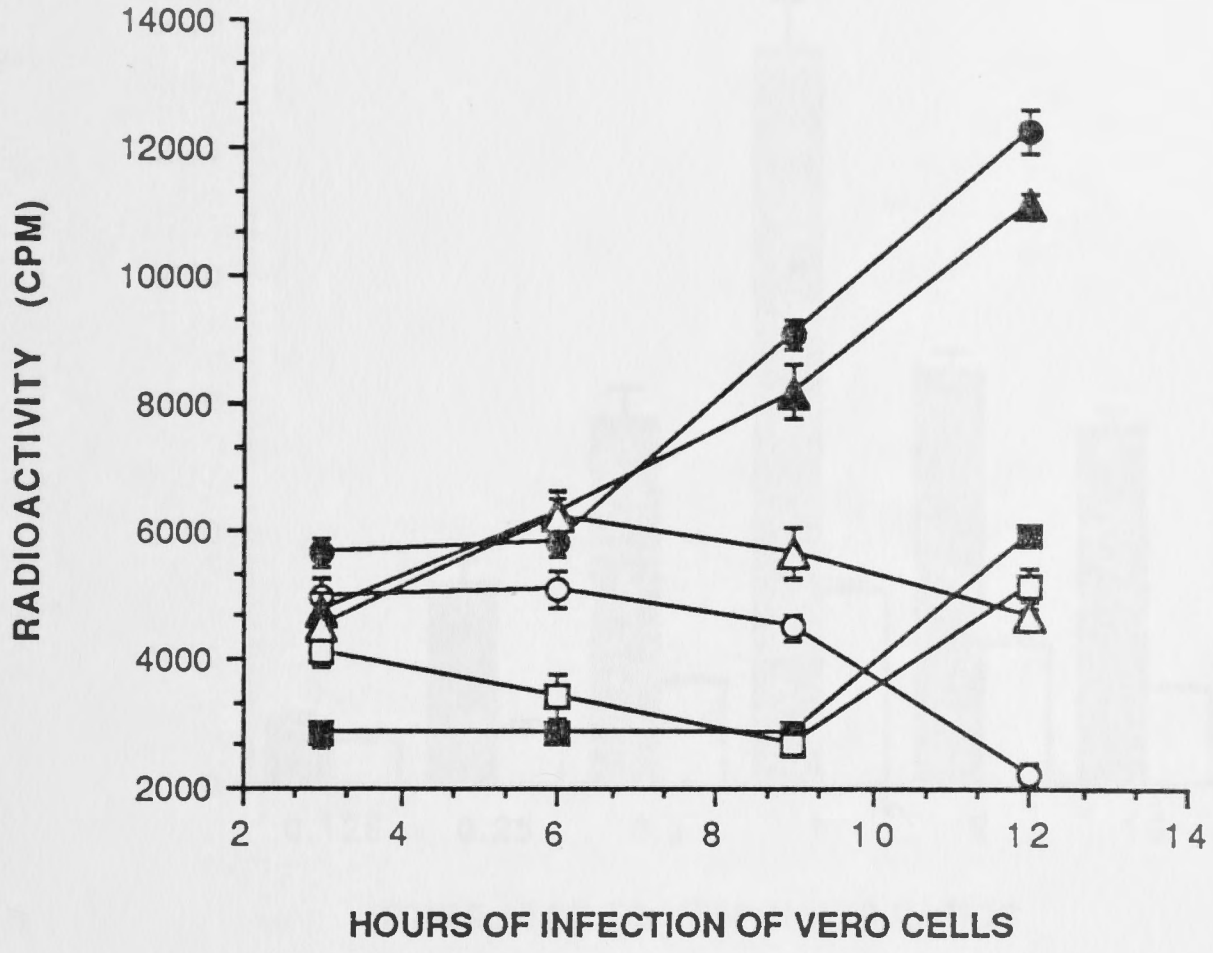


Fig. 2.2. Stimulation of WNV-primed splenocytes with lysates of Vero cells obtained at 3, 6, 9 and 12 h post-infection with WNV-V. $2x10^5$ enriched responder T cells from WNV-primed mice were stimulated with enriched B cells and dendritic cells at a 1:1 ratio, in the presence of UV-irradiated lysates of WNV-infected or uninfected control Vero cells at different dilutions for 5 days in a modified proliferation assay (see Materials and Methods). WNV-infected lysate (closed points) and control lysate (open points) were used either undiluted, (squares), 1/4 diluted, (circles) or 1/8 diluted, (triangles). Results represent the mean ^{3}H-TdR uptake by triplicate cultures ± S.E.M.

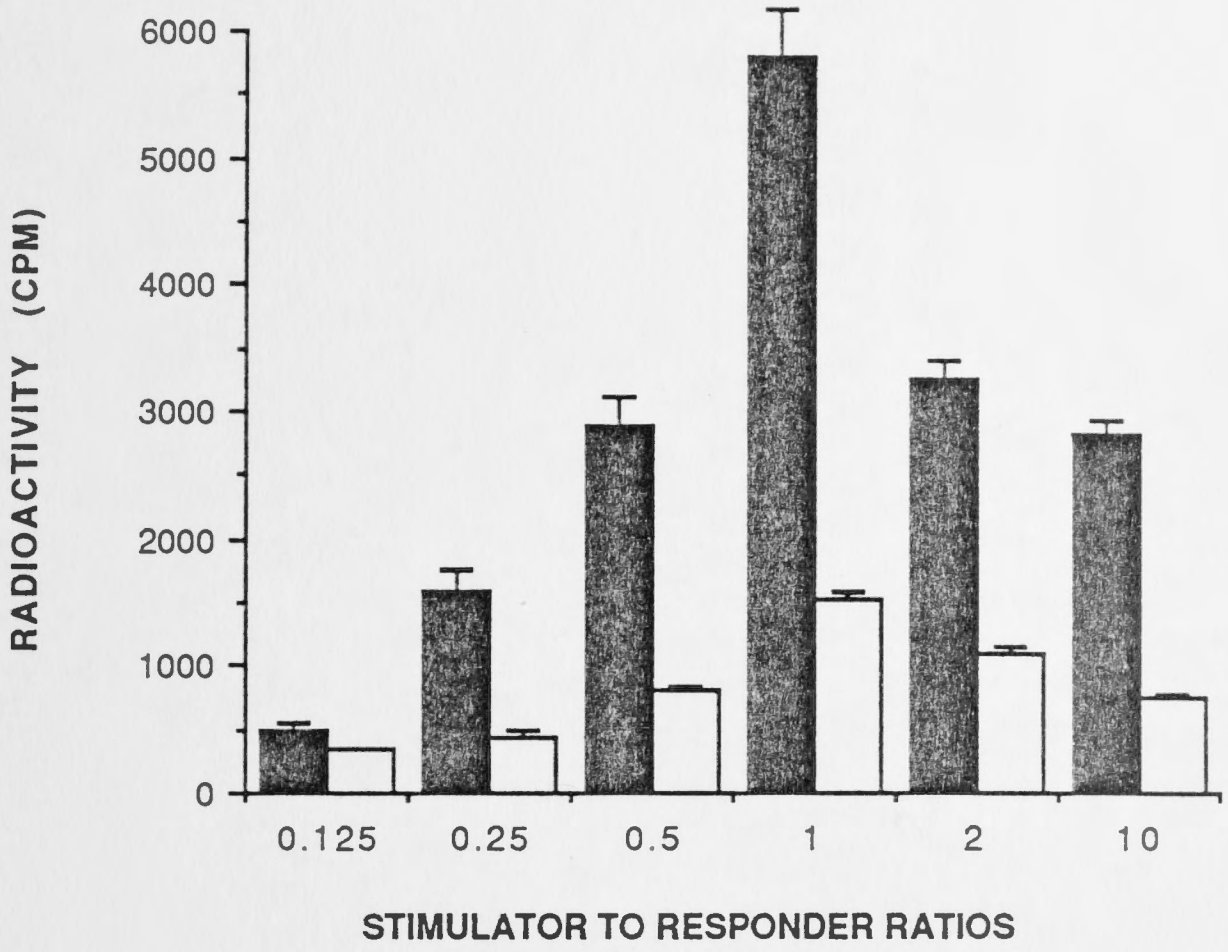


Fig. 2.3. Effect of stimulator to responder ratios on WNV-specific T cell proliferation. $2x10^5$ enriched $CD4^+$ T responders derived from mice primed i.v. with 10^6 pfu of WNV 14 days previously were co-cultured at different ratios with irradiated splenic APC enriched for B cells and dendritic cells. The APC were pulsed with WNV-infected Vero cell lysate stock (at a 1/4 dil.) (■) or control Vero lysate (□). Proliferation was assessed using the modified proliferation assay. Results represent the mean ^{3}H-TdR uptake by triplicate cultures ± S.E.M. from

Fig. 2.4.

Phenotype of APC populations. Each figure shows the distribution of fluorescence intensity in a sample of 10,000 cells. Cells were stained for Thy-1.2 and sIg markers prior to (A, B) and after (C, D) the depletion of macrophages and Thy-1.2$^+$ cells. Staining for sIg (A, C) was performed by using fluorescein-conjugated goat anti-mouse IgG antibodies. Dotted lines represent staining with control cells (see Materials and Methods). For Thy-1.2 staining, viable cells treated with biotinylated anti-mouse Thy-1.2 antibodies were stained with streptavidine R phycoerythrin conjugate. Dotted lines represent staining with streptavidine R phycoerythrine conjugate only. Prior to the enrichment procedures sIg$^+$ and Thy-1.2$^+$ cells constituted 65% (A) and 60% (B) respectively, of the total population. After the enrichment procedures, sIg$^+$ cells comprised 85% (C) and Thy-1.2$^+$ cells were almost absent (D).

Fig. 2·4

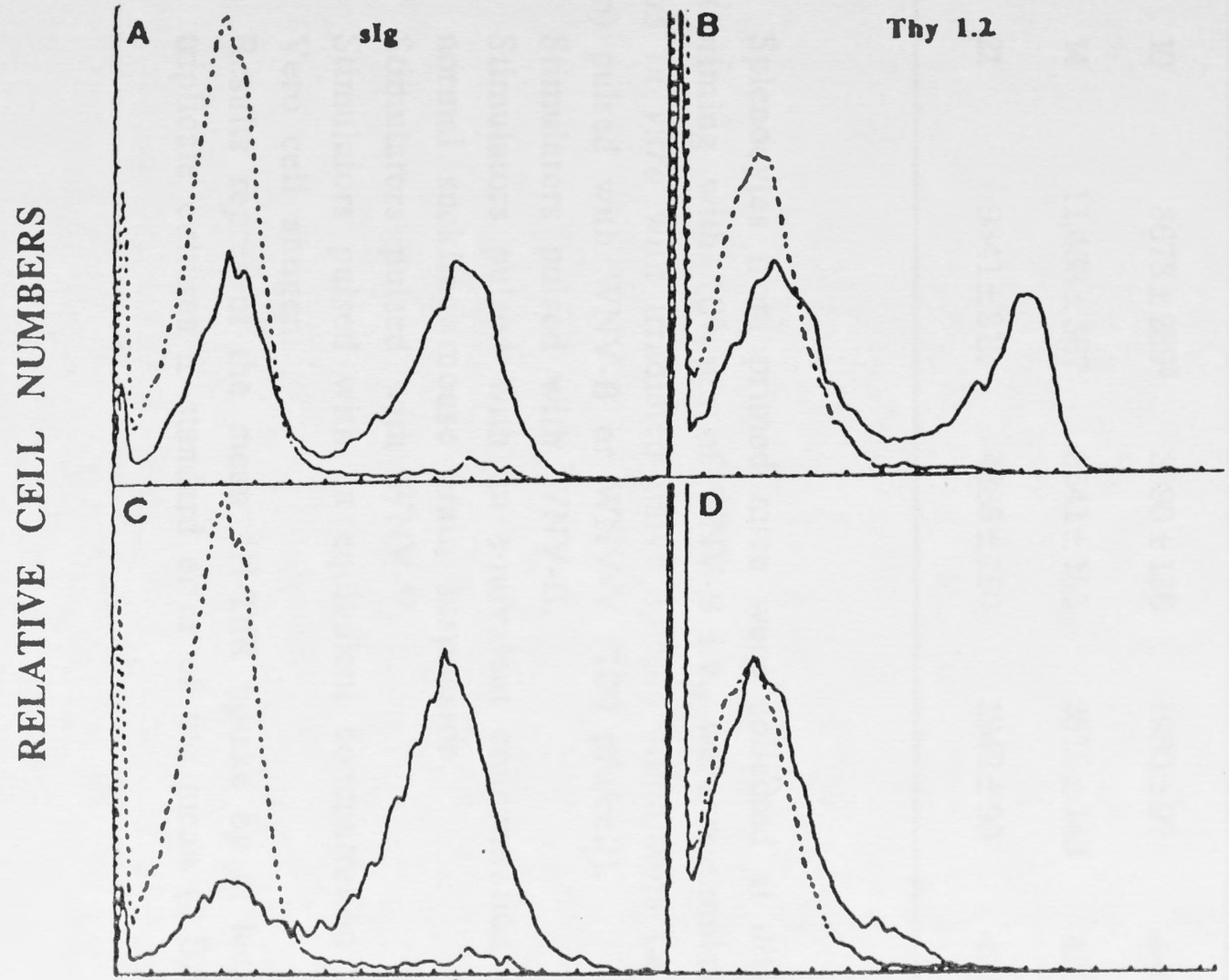

A
sIg
B
Thy 1.2
C
D
RELATIVE CELL NUMBERS
LOG FLUORESCENCE INTENSITY

Table 2.1 : *In-vitro* restimulation of WNV-immune splenocytes[a] with infectious preparations of WNV.

Days post priming	WNV-B		WNV-V	
	St + WNV-B[1]	St + SMB[2]	St + WNV-V[3]	St + Mo[4]
10	8673 ± 289[5]	3390 ± 136	1990 ± 97	4467 ± 285
14	11,463 ± 367	3541 ± 155	2518 ± 143	4575 ± 269
21	9841 ± 312	4365 ± 160	1942 ± 90	4916 ± 499

[a] Splenocytes from primed mice were collected at different days post-priming with 10^6 pfu of WNV-B i.v., were restimulated for 72 hours *in vitro* with irradiated naive splenic stimulators (at a 1:1 ratio) pulsed with WNV-B or WNV-V (100 pfu/cell).

1 Stimulators pulsed with WNV-B.

2 Stimulators pulsed with an equivalent concentration of normal suckling mouse brain suspension.

3 Stimulators pulsed with WNV-V.

4 Stimulators pulsed with an equivalent concentration of mock Vero cell antigen.

5 Results represent the mean ^{3}H-TdR uptake by at least triplicate cultures ± standard error of the mean (S.E.M).

Table 2.2: *In-vitro* restimulation of WNV-immune splenocytes[1] with UV-irradiated preparations of WNV.

Days post-priming	WNV-B		WNV-V	
	St.+WNV-B [2]	St.+SMB [3]	St.+WNV-V [4]	St.+Mo.Ag [5]
10	12,226 ± 255 [6]	5059 ± 159	14,082 ± 383	6928 ± 364
14	16,311 ± 365	3849 ± 103	17,387 ± 120	7119 ± 399
21	12,715 ± 272	4677 ± 167	13,650 ± 242	7015 ± 1075

1 Splenocytes from primed mice collected at different days post-priming with 10^6 pfu of WNV-B i.v., were restimulated for 72 hours *in vitro* with irradiated naive splenic stimulators (at a 1:1 ratio) in the presence of UV-irradiated preparations of WNV-B or WNV-V (equivalent to 100 pfu prior to irradiation).

2 Stimulators pulsed with UV-irradiated preparations WNV-B.

3 Stimulators pulsed with UV-irradiated preparations of normal suckling mouse brain suspension at an equivalent dilution.

4 Stimulators pulsed with UV-irradiated WNV-V.

5 Stimulators pulsed with UV-irradiated mock Vero cell antigen at an equivalent dilution.

6 As for Table 2.1

The data are representative of one of the two experiments performed.

Table 2.3: Virus specificity of T cell proliferation.

Responders[1]	APC + Ag	APC + Mo.Ag
WNV-primed	17,513 ± 1038[3]	531 ± 27
Influenza-primed[2]	1633 ± 74	473 ± 31
Naive	1241 ± 76	436 ± 75

1 $2x10^5$ enriched $CD4^+$ T cells, either from naive mice or those primed with WNV or influenza (A/WSN) 14 days previously, were restimulated with irradiated APC from 14 day WNV-primed mice at a 1:1 ratio, in the presence of 12 h Vero cell-derived, UV-irradiated WNV antigen (APC + Ag, at a 1/4 dilution) or mock control (APC + Mo.Ag)

2 The responses of influenza-primed $CD4^+$ T cells in presence of APC and A/WSN (10 HAU/well, in 50 µl aliquot) or control (allantoic fluid at an equivalent dilution) antigens were: 84,767 ± 942 and 1491 ± 149 cpm ± S.E.M. respectively.

3 As for Table 2.1

The data are representative of one of the two experiments performed.

Table 2.4

1 $2x10^5$ $CD4^+$ T cells from mice primed i.v. 14 days previously with 10^6 pfu of WNV were co-cultured with irradiated APC from the same mice at a 1:1 ratio, in the presence of 12 h Vero cell-derived, UV-irradiated WNV antigen (APC + Ag, at a 1/4 dilution) or mock control (APC + Mo.Ag).

2 Conventional assay: 3 days, either in 5% FCS or 1% NMS.

3 Modified assay: 3 days either in 5% FCS or 1% NMS, then 2 days in 8% EL-4 supernatant.

4 As for Table 2.1.

5 Figures in parenthesis indicate stimulation indices (SI), i.e., ratios of ^{3}H-TdR incorporation with APC + Ag to APC + Mo.Ag. SI were calculated from randomly selected pairs of APC + Ag and APC + Mo.Ag values within each group of observations. The mean SI $\pm$ S.E.M. from different groups were compared by Student's 't' test. SI with NMS differed significantly from those with FCS ($P< 0.0005$)

Table 2.4: Comparison of conventional and modified proliferation assays.[1]

Serum supplement of culture medium	Conventional assay [2]		Modified assay [3]	
	APC + Ag	APC + Mo.Ag	APC + Ag	APC + Mo.Ag
	Experiment I			
FCS-5%	5956 ± 431 [4] (3.27 ± 0.36) [5]	1853 ± 98	7022 ± 543 (3.12 ± 0.13)	2251 ± 89
NMS-1%	8991 ± 764 (11.3 ± 0.6)	795 ± 53	15,179 ± 1792 (17.8 ± 1.6)	848 ± 40
	Experiment II			
FCS-5%	7422 ± 88 (1.85 ±0.11)	4025 ± 196	21,219 ± 927 (2.03 ±0.12)	10,574 ± 1043
NMS-1%	2665 ± 245 (5.2 ± 0.23)	540 ± 26	11,649 ± 683 (12.9 ± 1.3)	865 ± 50

CHAPTER 3

FUNCTIONAL ANALYSIS OF MACROPHAGES, B CELLS AND SPLENIC DENDRITIC CELLS AS ANTIGEN-PRESENTING CELLS IN WEST NILE VIRUS-SPECIFIC MURINE T LYMPHOCYTE PROLIFERATION

Introduction

The ligand recognized by the T cell receptor of mature peripheral T cells is formed by the complexing of a peptide from a foreign protein together with a molecule of the MHC. Peptides associated with MHC class I molecules, present on the surface of most somatic cells, are generally recognized by the $CD8^+$ subset of T cells, whereas the $CD4^+$ subset recognizes peptides complexed with MHC class II molecules. In normal *in vivo* situations, class II MHC is expressed mainly on specialized lympho-reticular cells which function as APC for $CD4^+$ T cells.

The macrophage was the first APC to be identified (Ziegler and Unanue, 1981). In the past few years, a wide variety of other cell types have also been shown to function as APC. These include, B lymphocytes (Chesnut *et al.*, 1982), DC (Sunshine *et al.*, 1983; Inaba *et al.*, 1983), Kupffer cells (Ragoff and Lipsky, 1979), epidermal Langerhans cells (Stingl *et al.*, 1978), oligodendrocytes (Ting *et al.*, 1981), vascular endothelial cells (Hirschberg *et al.*, 1982), activated T cells (Brown *et al.*, 1984) and L cell fibroblasts transfected with MHC class II genes (Germain and Malissen, 1986). Thus a wide variety of diverse cell types which constitutively express, or can be induced to express, MHC class II molecules on their surface can potentially function as an APC.

The most extensively studied APC are macrophages, B cells and DC. These cells are likely to be the most important APC *in vivo* because they are located in secondary lymphoid tissues where T lymphocyte activation takes place. However these cells differ from each other in their relative quantity of expression of class II MHC antigens, in binding, uptake and processing of any

given antigen and in their secretion of co-stimulatory factors (reviewed by Harding *et al.*, 1989). These differences could influence the relative efficacy of these cells in the activation of subsets of $CD4^+$ T cells.

In Chapter 2, *in vivo* and *in vitro* conditions were described which were necessary for WNV-specific $CD4^+$ T cell proliferation in response to the presentation of WNV antigens by splenocytes from WNV-primed (14 day) mice depleted of macrophages and $Thy1.2^+$ cells. In this Chapter, the intrinsic antigen-processing and presenting ability of macrophages, B cells and SDC is examined by comparing their ability to stimulate the proliferative responses of WNV-immune $CD4^+$ T cells.

Materials and Methods

Mice:

Seven to 14 week-old female CBA/H ($H\text{-}2^k$), C57BL/6 ($H\text{-}2^b$), B10.T(6R) ($K^q\ I^q\ D^d$) and B10.AQR ($K^q\ I^k\ D^d$) mice were used. Mice were bred under specific pathogen free conditions at the JCSMR.

Virus:

As for Chapter 2.

Immunization of mice:

As for Chapter 2, except that in order to obtain higher frequencies of antigen-specific B cells, mice were immunized with four i.v. injections of 10^6 pfu of WNV at weekly intervals and B cell preparations were obtained one week after the final injection.

Vero cell-derived WNV antigen:

As for Chapter 2. This WNV antigen preparation was used at a 1/4 dilution in all the experiments unless otherwise stated.

Antibodies:

As for Chapter 2. Monoclonal anti-I-$A^{b,d,q}$ (M_5/114.15, IgG2b) and anti-I-A^k (11.52.1, IgG2b) antibodies were used as undiluted culture supernatant. Anti-Mac 1 (M 1/70) antibodies were used as neat culture supernatant. WNV-immune ascitic fluid was a gift from Dr.I.D.Marshall. IgG fractionation of this ascitic fluid was performed by using Staphylococcal protein A covalently linked to sepharose CL-4B (Protein A-Sepharose, Pharmacia, Sweeden), as described by Ey *et al* (1978). Protein A sepharose-purified IgG against influenza A/WSN was a gift from Dr.G.Ada.

Induction of peritoneal exudate macrophages:

Peritoneal exudate cells (PEC) were harvested (Beller *et al.*, 1980) 3 days after i.p. injection of mice with either 10^4 live *Listeria monocytogenes* (Beller *et al.*, 1980) in 1 ml of Hank's Balanced Salt Solution supplemented with 1% FCS, or 3 ml of 10% thioglycollate broth (Difco, Detroit., Mich.) and were cultured in DMEM supplemented with 1 % NMS, 2-ME and antibiotics. Cultures were incubated at 37^0 C in a humidified atmosphere of 5% CO_2 in air for 2 h, after which non-adherent cells were removed by thorough washing with DMEM. Adherent populations thus obtained have been shown to comprise 98-100% macrophages (Beller *et al.*, 1980).

In experiments designed to eliminate the possibility that DC were a minor contaminating population in *Listeria*-induced (LM) PEC and were responsible for a proportion of APC function of PEC, LM-PEC were plated directly in 96-well round-bottomed tissue

culture plates. The level of Ia expression on macrophages during overnight culture was maintained by heat-killed *Listeria* (hkLM, 10^6 bacteria in 200 µl of DMEM supplemented with 1% NMS) as described by Beller and Unanue (1981). After 14 h, PEC populations were vigorously washed three times with DMEM to remove non-adherent cells (which at this time would include DC) and hkLM before co-culturing remaining LM macrophages with responder $CD4^+$ T cells.

Preparation of B cells:

After initial culture of spleen cells on a plastic surface for 2 h in DMEM supplemented with 1% NMS to remove adherent cells such as macrophages and most DC, non-adherent cells were harvested and depleted of T cells and DC using optimal dilutions of anti-Thy-1.2 and 33D1 antibodies with rabbit serum as a source of complement. The resulting suspension contained 85% sIg^+ bearing cells as determined by FACS analysis (data not shown). Con A stimulation (5 µg/ml) of splenocytes prior to and after B cell enrichment indicated that > 98% of the proliferative response had been eliminated by the enrichment procedures (data not shown) indicating virtually complete depletion of T cells.

Mitogen-activated B cells:

B cells obtained from spleens of naive mice were seeded into 80 cm^2 tissue culture flasks at a density of $2x10^6$/ml in DMEM supplemented with 1% NMS. LPS and dextran sulphate (Sigma, St. Louis, MO, USA) were added at final concentrations of 50 and 20 µg/ml respectively. Cultures harvested on day 3, with about 40% of cells viable, mostly consisted of blast cells.

Generation of primary anti-I-A^k T cells:

A bulk culture of primary anti-I-A^k T cells was obtained by *in vitro* stimulation of B10.T(6R) (K^q I^q D^d) splenocytes ($4x10^6$/ml) with B10.AQR (K^q I^k D^d) splenocytes irradiated with 2000 rads from a ^{60}Co source, at a 1:1 ratio. These cells were cultured for 7 days in 100 ml of DMEM supplemented with 5% FCS in 80 cm^2 tissue culture flasks in a humidified atmosphere of 5% CO_2 in air at 37^0 C. After 7 days, the viable cells, separated on Isopac-Ficoll, were used as responders to evaluate the expression of MHC class II (I-A^k) antigens on macrophages.

Splenic dendritic cells:

Splenic DC were obtained from naive mice as described by Pereira *et al* (1986). Briefly, splenocytes in EMEM and 2-ME were seeded at $3x10^6$/ml in 185 cm^2 tissue culture flasks (Nunclon, Denmark) at 50 ml per flask. Cells were allowed to adhere for 2 h at 37^0 C in an atmosphere of 5% CO_2 in air, after which non-adherent cells were removed by thorough washing with EMEM. Adherent cells containing DC were then incubated in EMEM supplemented with 5% FCS, 2-ME and 25 mM Hepes. After 16 h incubation at 37^0 C, non-adherent or loosely adherent cells containing DC were harvested by gentle pipetting. These cells were then treated with anti-Thy1.2 and complement to remove contaminating T cells and rosetted with SRBC coupled by $CrCl_3$ with sheep anti-mouse IgG (Parish and McKenzie, 1978) to remove Ig^+ and FcR^+ cells. Dead cells and rosetted cells were removed by centrifugation on Isopac-Ficoll giving a viable cell population rich in DC. The yields of DC were 0.5% of the initial cell numbers.

Proliferation assay:

Proliferative T cell responses to WNV antigens were tested in the modified assay described in Chapter 2 using 12 h WNV-V-infected Vero cell lysates as the antigen source. In T cell proliferation assays, macrophage cultures used as APC were supplemented with $2x10^{-5}$ M of indomethacin (7 μg/ml of culture; Mizel, 1981) (Sigma, St. Louis, MO, USA) to eliminate the possible inhibitory effects of prostaglandins (PG) released by these cells (Humes *et al.*, 1977). Since the dry powder of indomethacin is sparingly soluble in the culture media or ethanol, the following method (Mizel, 1981) was found to be the most suitable for obtaining a clear solution. Ten milligrams of indomethacin was dissolved in 1.4 ml of 0.1% Na_2CO_3, followed by the addition of 12.6 ml of phosphate buffered saline (pH 7.2). The resultant solution was then used at a 1/100 dilution in the final cultures containing macrophages and T cells. B cells and DC were irradiated with 1000 and 2000 rads respectively, for use as APC.

Statistical analysis:

As for Chapter 2.

Results

Ability of macrophages to stimulate the proliferation of WNV-specific $CD4^+$ T cells:

Table 3.1 shows a comparison of the antigen-presenting function of LM macrophages and thioglycollate-induced (TH) macrophages from CBA/H mice to WNV-immune CBA/H ($H\text{-}2^k$) $CD4^+$ T cells in two representative experiments. In order to obtain optimal

responses, varying ratios of stimulator macrophage populations to responder T cells were used. LM macrophages, when pulsed with Vero cell-derived WNV antigen, consistently induced much higher proliferative responses than TH macrophages. The magnitude of the response was dependent upon the number of LM macrophages used, the highest responses being obtained with S:R ratios of 2.

B10.T(6R) anti-B10.AQR T cells (anti-Ia^k) were used to determine whether class II MHC expression was induced on LM macrophages and recognized by alloreactive T cells. Strong Ia^k-specific proliferation was stimulated by LM macrophages with Ia^k genes (CBA/H and B10.AQR) but not by B10.T(6R) (I^q) macrophages (data not shown).

To determine whether the WNV-specific APC function of CBA/H ($H\text{-}2^k$) macrophages was dependent upon the expression of class II MHC antigens, the effect of anti-Ia^k antibodies on WNV-immune, $CD4^+$ T cell proliferation was tested. The data presented in Table 3.2 shows that the presence of anti-Ia^k antibodies inhibited the APC function of macrophages whereas anti-$Ia^{b,d,q}$ antibodies had no effect.

Characterization of Ia positive adherent cell populations: The possibility that DC as a minor contaminating population in LM macrophages were responsible for the APC function of LM macrophages was tested. Adherent, LM-PEC depleted of DC overnight *in vitro* were prepared as described in Materials and Methods. The ability of these cells to present WNV antigens was compared with that of freshly isolated LM macrophages. As shown in Table 3.3, proliferative responses induced by DC-free LM macrophages cultured overnight, although lower, did not differ

significantly from freshly isolated macrophages, thus indicating that contaminating DC were not the major APC in fresh LM macrophage populations.

Adherent LM-PEC populations, after treatment with 33D1 antibodies plus complement, did not show significantly lower APC activity (Table 3.4). In contrast, depletion of mac-1^+ cells led to a substantial loss of APC function, indicating that the majority of T cell responses were induced by macrophages acting as APC.

Ability of B cells to induce WNV-specific proliferative T cell responses:

Preliminary studies indicated that B cells from mice primed with WNV 14 days earlier induced higher responses than those primed 7, 21 and 28 days earlier. Therefore, B cells obtained from such mice (B_{14}) were compared with B cells from naive mice (B_0) and mitogen-activated B cells for function as APC (Table 3.5).

B_0 cells were similar to B_{14} at a S:R ratios of 1 and 0.5, but at 0.25 their APC ability declined significantly, whereas that of B_{14} cells did not. Mitogen-activated B cells failed to function efficiently as APC in this system.

To test the possibility that the superior APC function of B_{14} cells at low S:R ratios was due to the presence of WNV-specific B cells, were made attempts to obtain higher frequencies of such B cells by immunizing four times with WNV (B^{++++}). Figure 3.1 shows the APC ability of B^{++++} cells, B_{14} and B_0 over a range of concentrations of WNV antigen. B^{++++} cells induced greater responses than B_{14} and B_0 at all antigen concentrations tested, except the 1/4 dilution, the optimal response being obtained at 1/8 dilution of antigen. At the highest antigen concentration (dilution 1/4),

B^{++++} cells showed suppression of T cell proliferation. Responses induced by B_{14} and B_0, in contrast, were antigen dose dependent with no suppression evident at high antigen concentrations, and a general decline in T cell responses as antigen concentration diminished. B_{14} cells were marginally better than B_0 cells.

Effect of antibodies to WNV on antigen-presenting function of LM macrophages and B^{++++} cells:

When LM macrophages were used as APC, addition of anti-WNV IgG antibodies resulted in a significant increase in the efficiency of presentation compared to control cultures supplemented with anti-influenza IgG or without antibodies (Table 3.6). In contrast, the addition of WNV antibodies to cultures of B^{++++} cells significantly reduced their ability to present antigen (Table 3.7).

Splenic DC as APC :

WNV-specific T cell proliferative responses following presentation of WNV antigen by SDC were evaluated using cells from C57BL/6 (H-2^b) and CBA/H (H-2^k) mice (Table 3.8). Responses of C57BL/6 T cells were highest with the highest numbers of SDC (S:R=1) plus WNV antigen and decreased as SDC numbers were reduced. Background proliferation was consistently low, regardless of SDC number. SDC from CBA/H mice induced a substantial level of background T cell proliferation at the two highest SDC levels (S:R=1 or 0.5) and the best WNV-antigen induced proliferation occurred at a S:R ratio of 0.5.

Comparative ability of macrophages, B cells and SDC to present WNV antigens to WNV-specific $CD4^+$ T cells:

LM macrophages, B cells from naive or 14 day WNV-primed C57BL/6 mice and SDC were each co-cultured with $CD4^+$ T cells

obtained from WNV-primed C57BL/6 mice at a 1:1 ratio. LM macrophages induced significantly higher proliferative T cell responses than the other APC which were generally similar (Table 3.9).

Discussion

In this study the efficacy of murine peritoneal macrophages, B cells and SDC to function as APC in secondary WNV-specific CD4+ T cell proliferative responses *in vitro* were evaluated. The results demonstrate that under appropriate conditions, all these cell types have the ability to function as APC. On a cell population basis LM macrophages were more efficient APC than SDC or naive and antigen-primed B cells.

Expression of class II MHC antigens is not constitutive in peritoneal macrophages but can be induced by certain stimuli such as *Listeria monocytogenes* (Beller *et al.*, 1980). The experiments designed to evaluate induced Ia expression on LM macrophages and its correlation with APC function *in vitro* showed that Ia-bearing macrophages were the only effective APC in the PEC populations. The procedures involving overnight adherence of PEC to plastic surfaces and anti-dendritic antibody and complement treatment would have significantly reduced contaminating DC, yet these treatments had no effect on the APC function of the LM-macrophages. Treatment with anti-Mac-1 antibody and complement, however, caused a substantial decrease in the APC function of the PEC. Furthermore, the finding that opsonization of WNV antigen by specific antibody resulted in an increased efficiency of antigen presentation (Table 3.6) together with the observation that DC do

not express FcR (Nussenzweig *et al.*, 1982), strongly argues against the possibility that DC might have contributed to the observed responses. Taken together, these findings suggest that the dominant functional APC in PEC were macrophages.

Normal resting B cells are poor presenters of soluble antigens, (Kakiuchi *et al.*, 1983). Inefficient antigen presentation by resting B cells has been ascribed to inadequate antigen uptake and processing (Chesnut *et al.*, 1982), low expression of Ia antigens (Monroe and Cambier, 1983) or glycosylation differences in expressed Ia molecules (Krieger *et al.*, 1988). In the case of antigen-specific B cells, binding of specific antigen to the surface Ig results in 10^3 to 10^4-fold more efficient presentation than presentation by non-Ig mediated pathways (Lanzavecchia, 1985). In the light of these reports, the APC function of B_0 and B_{14} and mitogen-activated B cells was evaluated. The finding that B_{14} cells induced higher responses than B_0 cells at a S:R ratio of 0.25 can be explained on the basis that frequency of antigen-specific B cells in B_{14} populations was likely to be higher than those from B_0 populations. To induce similar responses to B_{14} cells, B_0 populations (which take up the antigen predominantly by non-Ig pathway) would need higher numbers to generate the same concentration of ligand. This was possibly the case at a S:R ratio of 0.5.

Higher frequencies of WNV-specific B cells in splenic populations were attempted by giving weekly injections of WNV over a period of four weeks. The efficient APC function of such B^{++++} cells at a low concentration of antigen (Fig 3.1) together with the data showing the inhibition of this function by WNV-specific IgG (Table 3.7), strongly support the notion that specific sIg present

on these APC plays a role in antigen uptake in an epitope specific way. These observations are consistent with the view that continuous receptor cycling (Howard, 1985) allows specific B cells to accumulate and process antigen, thereby providing a higher concentration of ligand for the activation of T cells.

Even after repeated immunizations with WNV, which presumably gave a higher frequency of antigen-specific B cells in the splenic B cell populations (B^{++++}), the antigen-concentration required for optimal APC function of such cells was only two-fold lower than that required for B_0 or B_{14}. This contrasts with the findings of Lanzavecchia (1985), who showed that cloned antigen-specific B cells presented antigen to T cells at a 1000-fold lower concentration than non-specific B cells. Presumably, the present data from B^{++++} cells reflects the fact that B cell populations, even after hyperimmunization with WNV, contain only a tiny minority of WNV-specific B cells. Cloned WNV-specific B cell lines or hybridomas would be necessary for cell to cell comparison with macrophages and SDC.

Interestingly, with B^{++++} cells, a suppression of T cell proliferation was observed at the highest concentration of WNV antigen. Three different possibilities can be ascribed for such an effect. First, at high antigen concentration, specific B cells may generate a hyper-optimal concentration of ligand on their surface, which may, in turn, provide a negative signal for T cells (Matis *et al.*, 1983). This issue is investigated in Chapter 5. Secondly, specific antibodies secreted into the culture may decrease uptake of antigen by sIg, due to competition for antigen binding. Thirdly, higher concentrations of ligand displayed on the surface of these cells could be responsible for the activation

of inflammatory T cells (T inf or TH1), as described by Janeway *et al* (1988). T inf affect Th cells (TH2), a subset of $CD4^+$ T cells that help for specific antibody responses, by producing IFN-γ which can directly inhibit IL-4 action (Rabin *et al.*, 1986; Gajewska and Fitch, 1988). The latter lymphokine has been shown to be an autocrine growth factor for the Th subset (Mosmann *et al.*, 1986). Since both TH1 and TH2 have been shown to proliferate *in vitro* in response to antigenic stimulation, inhibition of TH2 by TH1 as an explanation for the present observation will be applicable only if the frequency of TH1 is much lower than TH2. Although murine B cells have been shown to express FcR for IgG and IgM (Basten *et al.*, 1972), unlike macrophages, B^{++++} cells failed to show enhanced APC activity in the presence of WNV-specific IgG. These differences may be explained as follows, 1) sIg-mediated uptake of antigen could be the major mechanism for B^{++++} cell uptake of antigen, and, 2) a potent phagocytic activity of macrophage APC provides an obvious advantage for rapid internalization of immune complexes via FcR (Segal *et al.*, 1983), which may not be the case with B cells.

Finally, SDC were tested for APC function. SDC from CBA/H mice induced high levels of background T cell proliferation at the two top SDC levels which obscured the antigen-specific responses. These findings could be due to a potent syngeneic mixed leukocyte reaction as reported by Nussenzweig and Steinman (1980). In contrast, the responses induced by DC from C57BL/6 mice were comparable with those obtained using B_{14} or B_0 APC. Inability of DC to induce the highest proliferative responses despite constitutively expressing high levels of MHC antigens

implies that the absence of potent phagocytic or pinocytic ability (Austyn, 1987) reduced their ability to induce T cell proliferation, at least in this *in vitro* system.

Taken together, these findings suggest that macrophages, B cells and SDC differ in their APC function in the stimulation of anti-WNV primed $CD4^+$ T cells *in vitro*. These differences could be due to, 1) their ability to take up antigens, 2) qualitative and quantitative difference in their expression of MHC class II antigens (Cullen *et al.*, 1981), 3) differences in their proteolytic machinery for antigen processing (Jensen, 1988; Harding and Unanue, 1988), and, 4) the presence or absence of specific antibodies. In the latter case, Manca *et al* (1985) showed that the conformational changes caused by the antibodies bound to antigen potentiated the presentation of antigen and further suggested that the recognition of such complexes by FcR of macrophages may represent a mechanism of self-potentiating the immune response. Taken together these observation suggest that, in secondary responses, macrophages and B cells may display different spectra of peptides from SDC.

If macrophages, B cells and SDC differ in their antigen-presenting function in the secondary *in vitro* stimulation then which type of APC predominate in the activation of resting T cells *in vivo* ? In an unprimed animal it is likely that both macrophages and B cells are in some state of activation due to on-going immune responses to gut flora or environmental antigens. Although macrophages are distributed widely in both lymphoid and non-lymphoid tissues (Unanue, 1984), they are rarely seen in T-dependent areas of lymph node and Peyer's patches, where T lymphocyte activation occurs. In the spleen,

macrophages are present in the marginal sinuses surrounding the periarteriolar lymphoid sheath and in the lymph nodes they are concentrated within the medullary sinuses (Hume *et al.*, 1983). Within the lymph nodes, B cells are primarily found in follicles (Sprent, 1973), but are rarely seen in non-lymphoid tissues. In contrast, DC are present in T-dependent areas of lymphoid tissues (Steinman and Cohn, 1973) and in most of the non-lymphoid tissues where antigens first gain access to the body. These three cell types also differ in their recirculation pattern which determines their ability to take up antigens from the tissues and to physically associate with T cells in the lymphoid organs. Since B cells recirculate from blood through follicles of lymphoid organs, and back to blood (Sprent, 1973), they may interact with T cells in T-dependent areas during their passage to follicles or within the follicle itself (which contain small numbers of $CD4^+$ T cells during secondary immune responses, Bhan *et al.*, 1981). Blood monocytes follow a similar pattern of recirculation (Hall and Morris, 1965). In contrast, DC are present in large numbers in afferent but not in efferent lymph (Drexhage *et al.*, 1979). Further, DC appear to traffic one-way from tissues to draining lymph node. This pattern of distribution of DC together with the constitutively expressed high levels of MHC antigens (Nussenzweig *et al.*, 1981) and their requirement in small numbers for T cell stimulation (Macatonia *et al.*, 1989) make them the most likely candidates for a major role in the primary activation of resting T cells. Their role in primary response *in vivo* is well-documented (Ron and Sprent, 1987; Lassila *et al.*, 1988). However, in the present study, DC were not as efficient as LM macrophages. It may be that due to their poor

phagocytic/pinocytic ability, they require an interaction with macrophages *in vivo*. The possibility that DC may bind to antigenic fragments released by macrophages has been suggested by Kapsenberg (1986).

In addition to their anatomic distribution as described above, further limitations on B cells to function as a predominant APC during a primary response are, 1) the low frequency of antigen-specific B cells in the naive animal, and 2) low levels of MHC class II antigens on resting B cells.

Although the macrophages from the periarteriolar lymphoid sheath engulf soluble circulating antigens, it is unlikely that these cells are involved in the activation of resting T cells, because they do not constitutively express MHC class II antigens and their chances of coming in contact with T cells are limited to T cells in transit from blood to periarteriolar sheath and vice-versa.

These findings suggest that *in vivo*, in the absence of specific antibody and antigen-specific B cells and limited class II MHC expression on macrophages, DC are likely to be important in the activation of resting T cells in primary immune responses. In the secondary immune response, germinal center B cells, antigen-specific B cells and class II^+ macrophages may also function as efficient APC.

In conclusion, the results presented in this Chapter indicate that peritoneal macrophages, when induced to express class II MHC antigens by *Listeria* (LM macrophages), are more potent APC than B cells or SDC for inducing *in vitro* proliferation of WNV-immune $CD4^+$ T cells. Moreover, SDC from CBA/H mice induced substantial levels of background proliferation, thus obscuring the evaluation of *in vitro* antigen-specific responses.

Time-consuming enrichment procedures together with low yields of SDC (0.8 to 1% of the total splenocytes) further limit the use of these APC for *in vitro* studies. Therefore, LM macrophages appear to be the best source of APC for *in vitro* evaluation of Ir gene effects of different MHC class II genes and responses against different flavivirus proteins, which will be investigated in the following Chapter.

Summary

In this Chapter, the relative efficacy of macrophages, B cells and SDC in presenting WNV antigens to WNV memory $CD4^+$ T cells was evaluated. The results indicate that, under appropriate conditions, all these cell types can function as APC. *Listeria*-induced (LM) peritoneal macrophages induced higher proliferative responses than SDC or B cells derived from naive or 14 day WNV-primed mice. The ability of LM macrophage populations to present antigen was specifically inhibited by anti-class II MHC antibodies.

On a cell population basis, B cells obtained from mice primed with WNV 14 days previously evoked higher responses than resting or mitogen-activated B cells. B cells from mice receiving weekly injections of WNV over a period of four weeks elicited optimal responses with lower doses of antigen than naive or 14 day WNV-primed B cells.

When macrophages were used as APC, addition of specific antibodies to WNV resulted in increased efficiency of presentation, probably due to increased uptake of antigen by opsonization. In contrast, addition of anti-WNV antibodies to

hyper-immune B cells reduced their efficacy presumably by reducing uptake of antigen by B cell sIg. When SDC from C57BL/6 mice were used as APC, WNV-specific proliferative responses were directly related to the number of stimulator cells used, whereas the higher levels of background proliferation shown by the SDC from CBA/H mice obscured the antigen-specific responses.

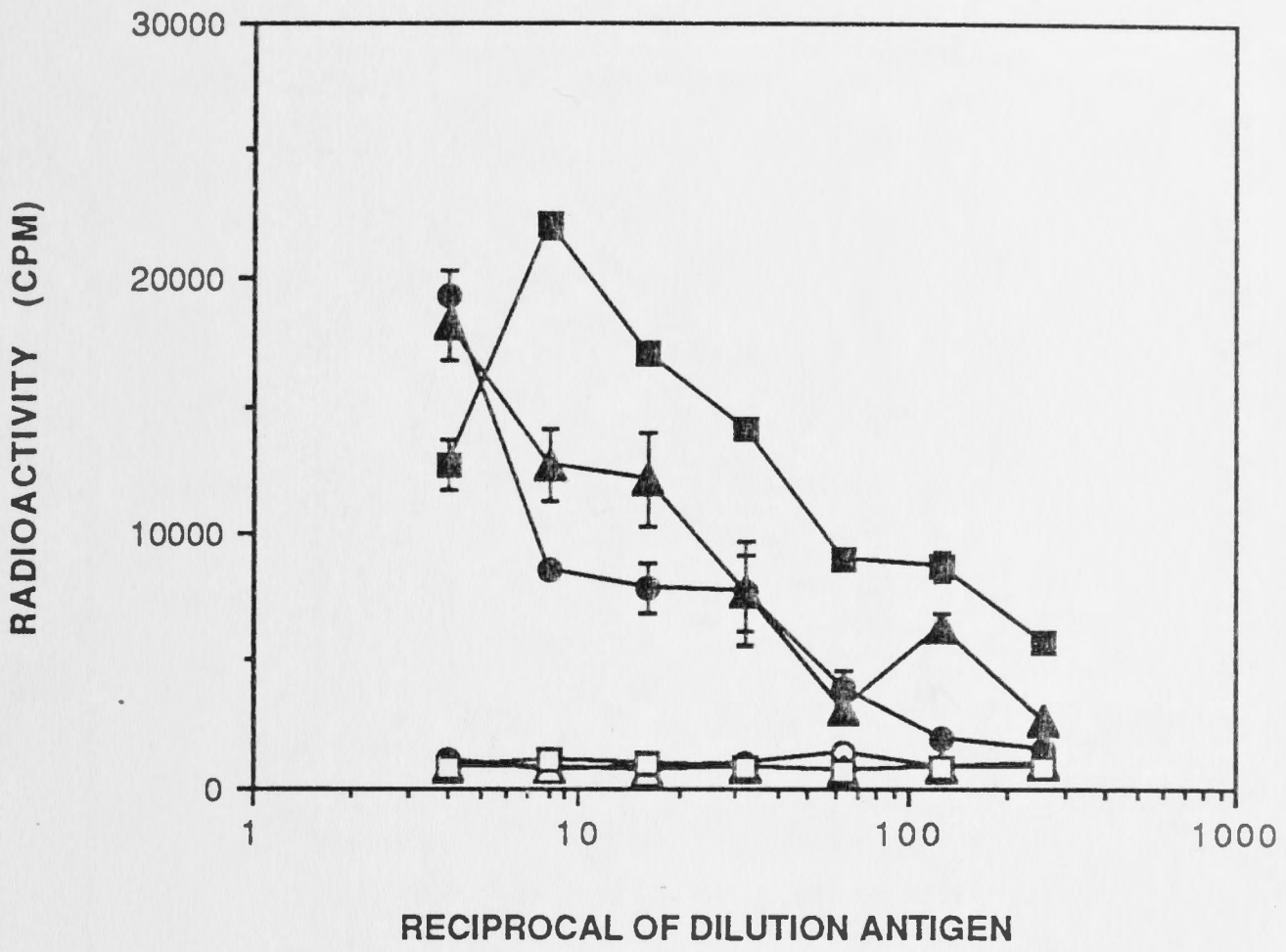


Fig 3.1 : APC function of B^{++++}, B_{14}, and B_0 cells over a range of WNV antigen concentration. $2x10^5$ $CD4^+$ T cells from 14 day WNV-primed mice were co-cultured with $2x10^5$ irradiated B cells in the presence of varying dilutions of WNV-antigen stock (closed points) or mock control (open points). B^{++++} (squares) were obtained from mice receiving 4 injections of WNV over a period of 4 weeks. B_{14} (triangles) cells were from 14 day WNV-primed mice and B_0 (circles) cells were from naive mice.

Table 3.1:

[1] $2x10^5$ $CD4^+$ T cells from 14 day-WNV-primed CBA/H mice were co-cultured with varying numbers of macrophages in the presence of a 1/4 dilution of WNV antigen stock (M + Ag) in the form of Vero cell lysate or mock control (M + Mo.Ag). Peritoneal exudate cells were collected 3 days after infection of either *Listeria monocytogenes* or thioglycollate broth. All the macrophage cultures were supplemented with indomethacin (7μg/ml).

[2] S:R ratios: stimulator to responder ratios.

[3] Results represent the mean ^{3}H-TdR (cpm) uptake by at least triplicate cultures ± S.E.M.

[4,5] Statistically significant ($P<0.05$).

[6,7] Statistically insignificant ($P>0.1$).

Table 3.1 : Antigen-presenting function of *Listeria*-and thioglycollate-induced peritoneal macrophages[1].

S:R ratios [2]	*Listeria*-induced macrophages		Thioglycollate-induced macrophages	
	M + Ag	M + Mo.Ag	M + Ag	M + Mo.Ag
Experiment 1.				
2	12,245 ± 746 [3]	806 ± 51	654 ± 58	488 ± 7
1	8340 ± 370	554 ± 38	601 ± 30	384 ± 38
0.5	2603 ± 162	299 ± 9	329 ± 23	208 ± 8
0.1	1015 ± 122 [4]	234 ± 7	683 ± 19 [5]	429 ± 31
Experiment 2.				
2	16,906 ± 629	1052 ± 45	666 ± 71	555 ± 19
1	9858 ± 402	680 ± 56	836 ± 29	503 ± 19
0.5	3515 ± 174	334 ± 21	395 ± 10	274 ± 13
0.1	1114 ± 57 [6]	335 ± 17	973 ± 49 [7]	536 ± 15

Table 3.2: Inhibition of antigen-presenting function of LM-macrophages by anti-Ia^k monoclonal antibodies [a].

Dilution of anti-Ia antibodies		Responses of CD4$^+$ T cells M+Ag	M+Mo.Ag
1/4	anti-Ia^k	882 ± 101 [2]	720 ± 36
1/10	anti-Ia^k	739 ± 56	430 ± 91
1/50	anti-Ia^k	1150 ± 182	647 ± 28
1/200	anti-Ia^k	2862 ± 560	923 ± 76
1/500	anti-Ia^k	3900 ± 490 [3]	813 ± 68
1/4	*anti-Ia^b	12,954 ± 1301 [4]	1385 ± 57
No antibodies		12,267 ± 906 [5]	1429 ± 43

[a] Enriched CD4$^+$ T cells ($2x10^5$) from WNV-primed CBA/H (H-2^k) mice were co-cultured with an equal number of CBA/H LM-macrophages in the presence of a 1/4 dilution of WNV antigen stock (M+Ag) or mock control (M+Mo.Ag) and varying dilutions of anti-Ia^k antibodies (IgG2b) made from hybridoma cell supernatant stock.

[3,4] Statistically significant ($P<0.0025$); [3,5] Statistically significant ($P<0.0005$),

[4,5] Statistically insignificant ($P>0.3$).

* Class-matched IgG2b antibodies

[2] As for Table 3.1

The data are representative of one of the two experiments performed.

Table 3.3: Effect of overnight culture and non-adherent cell removal[1] on ability of *Listeria*-induced peritoneal macrophage populations to act as APC to WNV-immune $CD4^+$T cells.

Stimulators	Responses of $CD4^+$T cells			
	Experiment I		Experiment II	
	M+Ag [2]	M+Mo.Ag [3]	M+Ag	M+Mo.Ag
LM-macrophages cultured 14h with hkLM	* 3831 ± 311 [4]	62 ± 0.8	4973 ± 233 [6]	194 ± 28
LM-macrophages cultured for 14h without hkLM	183 ± 27	123 ± 18	303 ± 42	79 ±8
Freshly harvested LM-macrophages	5180 ± 605 [5]	140 ± 20	5245 ± 548 [7]	141 ± 23

1 10^5 LM-macrophages from C57BL/6 mice were cultured for 14 hours in 96-well-round-bottomed plates with or without heat-killed *Listeria* (hkLM, 10^6 bacteria per well) and washed thrice with DMEM before the addition of WNV-immune $CD4^+$T cells ($2x10^5$) (S:R ratio = 0.5).

2 M+Ag: LM-macrophages pulsed with WNV antigen stock at a 1/4 dilution.

3 M+Mo.Ag: LM-macrophages pulsed with mock control.

4,5 Not significant ($P>0.05$)

6,7 Not significant ($P>0.3$)

* As for Table 3.1

Table 3.4: Phenotype of *Listeria*-induced peritoneal exudate cells[1].

PEC treatment	Responses of immune T cells	
	M+Ag	M+Mo.Ag
Untreated	*12,323 ± 806 [2]	750 ± 84
$33D_1$ + C	9524 ± 755 [3]	1717 ± 121
Mac-1+ C	1501 ± 112 [4]	1492 ± 94
Mac-1+ $33D_1$+ C	1371 ± 51	1499 ± 57
C only	11,232 ± 1008 [5]	432 ± 46

[1] 10^5 macrophages were plated for 2 h and successively treated with antibody and complement. Optimum dilution ($^{1}/_{16}$) of anti-dendritic antibody (33D1) used was determined previously by complement-dependent lysis of splenic dendritic cells. Anti-Mac-1 (M1/70) antibody was used as neat culture supernatants. After the depletion procedures, macrophages were co-cultured with $2x10^5$ WNV-immune $CD4^+$ T cells from C57BL/6 mice (S:R ratio = 0.5) in the presence of a 1/4 dilution of WNV antigen stock (M+Ag) or mock control (M+Mo.Ag).

[3,5] Statistically insignificant (P>0.1)

[4,5] Statistically significant (P<0.0005)

[2,5] Statistically insignificant (P>0.2)

* As for Table 3.1

The data are representative of one of the two experiments performed.

Table 3.5 : Ability of B cells to induce WNV-specific proliferative T cell responses [1].

	S.R Ratios					
	1		0.5		0.25	
	B + Ag	B + Mo.Ag	B + Ag	B + Mo.Ag	B + Ag	B + Mo.Ag
B_0 [2]	*2333 ± 407 [5]	289 ± 9	2724 ± 421 [7]	374 ± 55	1870 ± 59 [9]	370 ± 19
B_{14} [3]	2701 ± 170 [6]	297 ± 51	2633 ± 228 [8]	292 ± 34	2661 ± 427 [10]	213 ± 25
LPS/DexSO_4 [4]	563 ± 49	447 ± 34	442 ± 63	295 ± 15	357 ± 32	250 ± 14

1 Enriched $CD4^+$ T cells ($2x10^5$) from WNV-primed mice were co-cultured with varying numbers of irradiated (1000 rads) B cells in the presence of a 1/4 dilution of WNV antigen stock (B + Ag) or mock control (B + Mo.Ag).

2 B_0- B cells from naive mice; 3 B_{14}- B cells from 14 day WNV-primed mice;

4 LPS/DexSO_4- B cells from naive mice were cultured with lipopolysaccharide and dextran sulphate at a final concentration of 50 and 20 μg/ml respectively for 2 days; harvested and used as APC.

5,6 Statistically insignificant ($P>0.2$); 7,8 Statistically insignificant ($P>0.4$);

9,10 Statistically significant ($P<0.05$)

* As for Table 3.1.

The data are representative of one of the two experiments performed.

Table 3.6: Effect of antibodies to WNV on antigen-presenting function of macrophages [a].

Antibodies	Responses of CD4+T cells	
	M+Ag	M+Mo.Ag
Anti-WNV IgG	*36,023 ± 2590 [1]	2480 ± 154
Anti-influenza IgG	17,397 ± 1493 [2]	2600 ± 104
No antibodies	14,906 ± 1371 [3]	2089 ± 176

[a] $2x10^5$ $CD4^+$ T cells from WNV-primed C57BL/6 mice were co-cultured with 10^5 LM-macrophages in the presence of a 1/4 dilution of WNV antigen stock (M+Ag) or mock control (M+Mo.Ag). WNV-specific and control (influenza-specific IgG) antibodies were added at the initiation of cultures at a final concentration of 2 mg/ml in 50 µl aliquots. Macrophage to responder ratio = 0.5.

[1,2] Statistically significant ($P<0.0025$)
[2,3] Statistically insignificant ($P>0.1$)
[1,3] Statistically significant ($P< 0.0005$)
* As for Table 3.1
The data are representative of one of the two experiments performed.

Table 3.7: Effect of antibodies to WNV on antigen-presenting function of B^{++++} cells [1].

Antibodies	Responses of $CD4^+$ T cells [2].	
	B^{++++} + Ag	B^{++++} + Mo.Ag
Anti-WNV IgG	*15,147 ± 1097 [3]	1131 ± 42
Anti-influenza IgG	25,557 ± 1386 [4]	1500 ± 58
No antibodies	24,928 ± 1617 [5]	1926 ± 38

1 B^{++++} cells were obtained from the mice receiving weekly injection of WNV over a period of four weeks.

2 $2x10^5$ $CD4^+$T cells from WNV-primed C57BL/6 mice were co-cultured with $5x10^4$ irradiated B^{++++} cells in the presence of a 1/8 dilution of WNV antigen stock (B^{++++} + Ag.) or mock control (B^{++++} + Mo.Ag). WNV-specific and control (influenza-specific) antibodies were added at the initiation of cultures at a final concentration of 2 mg/ml in 50 µl aliquots. B^{++++} cells to responder ratio = 0.25

3,4 Statistically significant ($P<0.0025$)

3,5 Statistically significant ($P<0.0005$)

4,5 Statistically insignificant ($P>0.3$)

* As for Table 3.1

The data are the representative of one of the two experiments performed.

Table 3.8: WNV-specific T cell proliferation in response to the presentation of WNV-antigen by splenic dendritic cells [a].

S:R ratios	Responses of $CD4^+$T cells	
	SDC+Ag	SDC+Mo.Ag
	SDC from C57BL/6 mice	
1	5199 ± 257 [1]	1014 ± 113
0.5	2461 ± 139	1048 ± 129
0.25	2395 ± 69	920 ± 58
	SDC from CBA/H mice	
1	4617 ± 412 [2]	3223 ± 208 [3]
0.5	7105 ± 517 [4]	4048 ± 362 [5]
0.25	1706 ± 24 [6]	1039 ± 40 [7]

[a] $2x10^5$ $CD4^+$T cells from WNV-primed mice were co-cultured with varying numbers of irradiated syngeneic SDC in the presence of a 1/4 dilution of WNV antigen stock (SDC+Ag) or mock control (SDC+Mo.Ag).

[2,3] Statistically significant ($P<0.025$).

[4,5] Statistically significant ($P<0.0025$).

[6,7] Statistically significant ($P<0.0005$).

[1] As for Table 3.1

The data are representative of one of the two experiments performed.

Table 3.9 : Relative efficacy of different types of antigen presenting cells [a].

APC	Responses of $CD4^+$ T cells.	
	APC + Ag	APC + Mo.Ag
LM-macrophage	*14,880 ± 531 [1]	246 ± 17
B_0	7,952 ± 327 [2]	170 ± 13
B_{14}	9,037 ± 420 [3]	145 ± 4
SDC	7,743 ± 304 [4]	179 ± 16

[a] $2x10^5$ $CD4^+$ T cells from WNV-primed C57BL/6 mice were co-cultured with equal numbers of 1) LM-macrophages - Listeria-induced macrophages, 2) B_{14} - B cells from 14-day WNV-primed mice, 3) B_0 - B cells from naive mice, and 4) SDC - splenic dendritic cells; in the presence of a 1/4 dilution of WNV antigen stock (APC + Ag) or mock control (APC + Mo.Ag).

1,2; 1,3 & 1,4 Statistically significant ($P< 0.0005$).

* As for Table 3.1

The data are representative of one experiment.

CHAPTER 4

IMMUNE RESPONSE GENE CONTROL OF MHC CLASS II-RESTRICTED T CELL RESPONSES TO WEST NILE AND KUNJIN VIRUS

Introduction

The capacity of an individual to respond to an antigenic stimulus is in part genetically determined.

MHC-linked immune response (Ir) genes encode cell surface molecules that restrict the specificity of T lymphocyte responses to antigen (Benacerraf, 1978). $CD4^+$ T cells, upon activation, secrete lymphokines that influence a variety of functions, such as specific antibody secretion by B cells (IL-4, 5 and 6), differentiation of Tc cells (IL-2, 4 and 6), inhibition of viral replication (IFN-γ, tumour necrosis factor) and macrophage activation (IFN-γ). In addition, $CD4^+$ T cells are also involved in the initiation of inflammatory responses. Antigen recognition by $CD4^+$ T cells is restricted by class II MHC gene products present on the surface of APC such as DC, macrophages and B cells. The epitope recognized is formed by a peptide of 6 to 20 amino acids derived from a foreign antigen complexed with class II MHC (Babbitt *et al.*, 1985; Buus *et al.*, 1986). Amino terminal domains of both chains of class II MHC molecules form a binding site for peptides (Brown, 1988). Most of the polymorphic residues amongst different MHC alleles are found in and around this binding site. The influence of MHC and background genes can be studied in inbred mouse strains to give insight into potential problems with viral vaccines in human populations.

$CD4^+$ T cell recognition of non-structural, cytosolic and structural, membrane-associated proteins, has been shown in several virus infections. In the case of influenza A, immune Th cells from BALB/c ($H\text{-}2^d$) mice recognize purified protein

preparations such as haemagglutinin, neuraminidase, matrix and nucleoprotein (Hurwitz *et al.*, 1985). Hepatitis B virus-specific, HLA-DPW4-restricted human T cell lines of helper/inducer class were found to respond strongly to epitopes derived from hepatitis B surface antigen (Celis *et al.*, 1988b). In the case of rabies virus, human T cells restricted by HLA-DR7 were shown to be specific either for glycoprotein or ribonucleoprotein (Celis *et al.*, 1988a). Similarly, dengue-immune Th cells from BALB/c mice have been shown to recognize C, prM, E, NS1 and NS2 proteins (Rothman *et al.*, 1989).

Some flaviviruses cause disease and vaccines are needed as reviewed in Chapter 1. West Nile and Kunjin virus infections can be studied in mice but thus far, T lymphocyte responses to flaviviruses under the influence of different MHC haplotypes and genetic backgrounds have been little studied (Hill, 1990). Kunjin virus is a member of the serological complex represented by Japanese encephalitis virus, Murray Valley encephalitis virus, West Nile virus and St. Louis encephalitis virus. This virus is widely distributed in Australia, often in association with Murray Valley encephalitis virus (Marshall *et al.*, 1982) but is rarely associated with clinical infection (Muller *et al.*, 1986). West Nile virus, which is widely distributed in different parts of the world, shares 93% homology with Kunjin in the deduced amino acid sequence; which is far more than the other characterized members of this group (Coia *et al.*, 1988).

The present study analyzes the murine $CD4^+$ T cell response to West Nile and Kunjin virus, identifies the major virus antigens recognized and demonstrates that the response is influenced by MHC class II and background genes.

Materials and Methods

Mice:

Specific pathogen free mice, bred at the JCSMR were used at 8 to 10 weeks of age. H-2 haplotypes and the abbreviations of the strains used in this Chapter are shown in Table 4.1.

Viruses:

Flaviviruses: West Nile, Sarafend strain and Kunjin-strain MRM 16 were obtained from Dr.I.D.Marshall, and grown in 4 day-old suckling mouse brains as described in Chapter 2. These virus stocks were titrated by plaque assay on Vero cells.

Kunjin-vaccinia virus (Kunjin-VV) recombinants: A series of vaccinia virus recombinants used in this study, containing various segments of Kunjin cDNA (Parrish *et al.*, unpublished) under the control of vaccinia early-late promotor P7.5, within the thymidine kinase region of the vaccinia genome, are shown in Fig. 4.1. The 5' end of the Kunjin genome, encoding sequences for C, prM/M, E, NS1, NS2A, NS2B and 75 amino acid residues from the aminoterminus of NS3, is encoded by VKV 1031. VKV 1022, encodes 78 amino acid residues from the carboxyterminus of NS4B and all of NS5. VKV 1023, encodes 20 amino acid residues from the carboxyterminus of NS2B, all of NS3 and 85 residues from the aminoterminus of ns4a. VKV 1024, spanning all of NS3-NS5, was prepared from 1023 and 1022 by addition of the bridging sequences encoding ns4a and NS4B.

The thymidine kinase-negative derivative of the WR strain of vaccinia virus (VVTK$^-$), which was used as a control for obtaining CV1 cell lysates (see below), was a gift from Dr.D.Boyle.

Virus stocks were prepared from infected CV-1 cells (0.01 pfu/cell) grown in 2 litre acid washed roller bottles (Schott, Mainz, Germany). Titrated stocks were stored in 100 µl aliquots in sterile vials at -70^0 C.

Titration of Kunjin-vaccinia virus recombinants:

Stock virus preparation was incubated with an equal volume of trypsin (1 mg/ml) in gelatin saline for 30 min at 37^0 C. After neutralization of the trypsin with 800 µl of saline containing 5% FCS, serial 10-fold dilutions were made and the virus titrated as plaques grown on human 143B cell monolayers in 6 well Linbro plates (Flow Laboratories, Inc, McLean, VA). Virus was adsorbed for 1 h before the cell monolayers were overlaid with EMEM containing 2.5% FCS. After a 2 day incubation period at 37^0 C in a humidified atmosphere containing 5% CO_2 in air, monolayers were fixed and stained with 0.1% crystal violet in 20% ethanol for 5 min, dried and plaques counted.

Immunization of mice:

Mice were immunized i.v. with 10^6 pfu of WNV or Kunjin-VV (or with VVTK$^-$ control) diluted in 200 µl of gelatin saline (0.5% W/V gelatin in borate buffered CaMg saline, pH 7.2). Before use for immunization, vaccinia virus stocks were diluted in 2 ml of gelatin saline to contain the desired pfu and sonicated as (pp.59) described in Chapter 2.

Vero cell-derived WNV antigen:

As for Chapter 2. This WNV antigen preparation was used at a 1/4 dilution in all the experiments.

Vero-cell derived Kunjin antigen:

Kunjin antigens were prepared using Kunjin infected Vero cells as described for WNV in Chapter 2. This Kunjin antigen preparation was used at a 1/4 dilution (optimal dilution).

Recombinant vaccinia virus-derived Kunjin antigens:

CV1 cells grown in 2 litre acid-washed roller bottles (Schott, Mainz, Germany) in EMEM supplemented with 5% FCS were infected for 1 h at 37^0 C with VKV 1024, VKV 1023, VKV 1022, VKV 1031 or VVTK- viruses at a multiplicity of 0.02 pfu per cell, using 10 ml of EMEM supplemented with 5% FCS. After 1 h unadsorbed virus was removed by 2 washings with EMEM and monolayers were cultured at 37^0 C in EMEM supplemented with 2% FCS. At 24 h post-infection, monolayers were washed twice with EMEM, 15 ml of EMEM was added to each roller bottle and the bottles were stored at -70^0 C. Freeze-thawed lysates were sonicated (P. 59) as described in Chapter 2. These lysates, used at a 1/4 dilution (optimal dilution) without UV-irradiation, hereafter will be referred to as 1024, 1023, 1022 and 1031 or recombinant vaccinia virus-derived Kunjin antigens.

Antigen presenting cells:

Two types of APC used in the present study were, 1) LM-macrophages, obtained 3 days after i.p. injection of live *Listeria monocytogenes* (described in Chapter 3), and, 2) B cells obtained from 14 day-primed (WNV or Kunjin) mice (described in Chapter 3).

In the T cell proliferation assays, macrophage cultures were supplemented with $2x10^{-5}$ M indomethacin (7 µg/ml) as described in Chapter 3. B cells were irradiated with 1000 rads from a ^{60}Co source before using them as APC.

Generation of primary anti-Ia^k T cells:

As for Chapter 3.

Proliferation assay:

Proliferative T cell responses to WNV and Kunjin antigens were tested in the modified assay described in Chapter 2 using 3 different sources of antigens, 1) WNV-V-infected Vero cell lysates, 2) Kunjin-infected Vero cell lysates, and 3) recombinant vaccinia virus-derived Kunjin antigens.

Statistical analysis:

As for Chapter 2.

Results

The responses of immune T cells are dependent upon the type of APC:

In the initial experiments, WNV-and Kunjin-immune CD4$^+$ T cells from mice of different MHC haplotypes and backgrounds were restimulated *in vitro* using H-2 compatible macrophages and B cells as APC in the presence of WNV and recombinant vaccinia virus-derived Kunjin antigens.

a) Responses to WNV antigens: In the H-2^k haplotype, the responses induced by macrophage APC were higher than those induced by B cells (Table 4.2). CBA macrophages induced higher responses from H-2-matched T cells (of either CBA or BR origin) than BR macrophages. On the other hand, B cells from both CBA and BR showed low but equal stimulatory capacity. BR T cells responded more weakly than CBA T cells, given the same APC.

In the case of the H-2^b haplotype, B cells from B/b and B6 were both efficient APC, comparable to B6 macrophages and

superior to B/b macrophages. T cells from both B/b and B6 showed similar responses when B cells were used as APC, but B/b macrophages seemed less active as APC than B6 macrophages.

B cells from the H-2^q haplotype invariably induced higher responses than macrophages. D/1 macrophages induced high background proliferation.

These results indicate that H-2 type, genetic background and APC type may all affect the proliferation assay.

b) Responses to recombinant vaccinia virus-derived Kunjin antigens: Tables 4.3 to 4.5 show the responses of Kunjin-immune T cells from the H-2^k, H-2^b and H-2^d haplotypes respectively, from one representative experiment, using macrophages and B cells as APC and lysates of CV1 cells infected with various recombinant vaccinia viruses to identify Kunjin proteins that give rise to T cell epitopes on APC.

In the case of the H-2^k haplotype (Table 4.3), both the CBA and BR strains gave T cells responsive to 1031 and 1022, the former being stronger. Macrophage APC induced higher T cell proliferation with the VVTK$^-$ control than B cells, but the pattern of response was generally similar for both types of APC.

With the H-2^b haplotype (Table 4.4), T cells from both B/b and B6 responded to 1031, 1022 and 1024 in decreasing order of strength. As before, higher background proliferation with macrophages pulsed with VVTK$^-$ antigen lysates obscured responses to some extent. In contrast in Table 4.2, B/b macrophages were better APC than B6 in the case of 1031. The higher backgrounds with VVTK$^-$ were also evident with macrophage APC from H-2^d strains (Table 4.5). Also, D2 macrophages induced no responses with 1031, whereas the latter

induced strong responses when B/c macrophages were used. However, the general pattern of responses was basically similar to that seen with H-2^k, viz. responses with 1031 and 1022, not with 1023 and 1024.

Expression of class II MHC antigens on LM-macrophages from different mouse strains:

Macrophages from BR, D/1 and 6R mice stimulated low T cell responses against WNV antigens. In order to explore the possibility that this could be due to low levels of MHC class II antigens on LM macrophages, the ability of macrophages from CBA and BR mice to restimulate *in vitro* proliferation of alloreactive anti-Ia^k T cells was compared. TH peritoneal macrophage populations, which do not express high Ia^+ antigen levels (Beller *et al.*, 1980) were used as a control.

LM macrophages from both CBA and BR mice induced lower T cell responses at S:R ratios of 1 than at 0.25 or 0.125 (Table 4.6). TH macrophages also showed a similar inverse dose response but the magnitude of response was lower. Peak responses were similar with CBA and BR macrophages. Similar results were obtained when LM macrophages from D/1 and 6R were tested with anti-Ia^q T cells (data not shown).

These results indicated that LM macrophages from BR, DBA and 6R mice were not deficient in the levels of expressed MHC class II antigens, but raised an alternative possibility that the lower responses could be caused by suppression of T cell proliferation when high numbers of macrophages were used as APC. Variation between different batches of LM macrophages with respect to dose response might account for differences in strain comparison as noted for B6 and B/b in Tables 4.2 and 4.3.

Therefore the dose responses of stimulator macrophages from different mouse strains were investigated to further evaluate the effect of class II MHC Ir genes on $CD4^+$ T cell responses to WNV and Kunjin antigens.

Evaluation of Ir gene effects under optimal stimulatory conditions with macrophages as APC:

a) Responses to WNV antigens: Fig. 4.2 shows the responses of immune T cells from H-2^b, H-2^d, H-2^k and H-2^q haplotypes to WNV antigens using varying numbers of H-2-matched macrophages as APC from one of the two representative experiments. Macrophages from B/b, B/c, and CBA mice were more efficient APC for H-2-matched T cells than those from B6, D2 and BR mice. T cells from B/c, CBA and B/b mice were also better responders than B6 and BR T cells. With the H-2^q haplotype, D/1 T cells were better responders than 6R T cells and were more efficiently stimulated by D/1 macrophages than 6R APC. Macrophages from the strains with C57BL background induced suppression of T cell proliferation at high stimulator cell numbers. B6 macrophages induced higher T cell responses at the lowest cell numbers tested. Therefore the possibility that these macrophages may induce optimal responses at even lower S:R ratios was evaluated, but the results indicated that the best T cell responses were at S:R of 0.125 and lower APC numbers did not induce higher responses (data not shown).

b) Responses to recombinant vaccinia virus-derived Kunjin antigens:

Figures 4.3, 4.4 and 4.5 show the responses of $CD4^+$ T cells from Kunjin-primed H-2^k, H-2^b and H-2^d mice against recombinant vaccinia virus-derived Kunjin antigens using varying numbers of

macrophages as APC from one of the two representative experiments. With the H-2^k haplotype (Fig. 4.3) responses to 1022 were always above the VVTK$^-$ control, responses to 1031 were usually above the control (9 out of 12 data points), though not as high as 1022, but only 1 out of 12 data points were above the VVTK$^-$ control in the case of 1023 and 1024. There was no clear pattern of dose response with varying macrophage numbers. In contrast, in the case of H-2^b (Fig 4.4) and H-2^d (Fig 4.5) higher numbers of macrophages generally suppressed responses and maximal proliferation occurred at S:R ratios of 0.25 or 0.5. Also, with both of these haplotypes, responses to 1031 were generally higher than those to 1022, with one exception in each case. Responses to 1024 and 1023 were never high, though occasionally above the VVTK$^-$ control. Despite the manipulation of macrophage numbers, responses of H-2^b T cells to 1024 presented by B cell APC (Table 4.4) were not achieved with macrophages as APC.

Induction of memory specific for different Kunjin proteins:

The ability of Kunjin-VV recombinants to prime for a secondary *in vitro* proliferative response was evaluated by priming CBA mice with these viruses. CD4$^+$ T cells obtained 3 weeks later were stimulated with LM macrophages pulsed with Vero cell-derived Kunjin antigen. Although the high background proliferation obscured responses to some extent, priming with VKV 1031 induced significantly higher Kunjin-specific T cell responses than priming with the whole virus, whereas the response of VKV 1022-immune T cells were comparable with Kunjin-immune T cells (Table 4.7). VKV 1023-and 1024-immune

T cells did not give statistically significant responses to Vero cell-derived Kunjin antigens.

Discussion

In this Chapter, the $CD4^+$ T cell responses to WNV and recombinant vaccinia virus-derived Kunjin antigens presented by macrophages and B cells were analyzed. Since macrophages and B cells differ in their ability to take up and process exogenous antigens (discussed in Chapter 3), it was possible that these different APC may display different epitopes to T cells.

Recombinant vaccinia viruses encoding foreign genes provide a means to study the immunobiology of encoded proteins. The flavivirus genome consists of a single strand RNA of positive sense, and is about 11 kilobases in length. In an infected cell, the translation of this RNA initiates with the capsid protein near the 5' end of the genome and proceeds sequentially through the genome to produce one precursor polyprotein. Individual proteins are produced by post-translational cleavage of this polyprotein on specific sites (Rice *et al.*, 1986). The cleavage products of the polyprotein include, three structural (C, prM and E) as well as large non-structural polypeptides (NS1, NS3 and NS5), along with four small non-structural polypeptides (ns2a, ns2b, ns4a and ns4b). Structural proteins have been implicated in haemagglutination and neutralization, whereas NS1 has been shown to be a soluble complement-fixing antigen (Smith and Wright, 1985). In the present study, recombinant vaccinia virus vectors encoding the various portions of Kunjin genome were used, but whether cleavage of such encoded proteins

occurred as in natural flavivirus infection is not known. Construct VKV 1022 encodes NS5 and a portion of NS4B, VKV 1023 encodes NS3 and portions of ns4a and NS2B, VKV 1024 encodes all of the NS3-NS5 and a part of NS2B, while VKV 1031 encodes C, M, E, NS1, NS2A, NS2B and a part of NS3.

The present experiments led to several interesting findings. Despite many potential T cell determinants encoded by the Kunjin virus genome, the expressed T cell repertoire appeared to be focussed on a limited number of immunodominant sites. This has also been shown to occur with other protein antigens, such as cytochrome C (Solinger *et al.*, 1979), hen-egg lysozyme (Maizels *et al.*, 1980), and influenza haemagglutinin (Hackett *et al.*, 1983).

The results presented in Figs. 4.3, 4.4 and 4.5 together with those in Table 4.7 strongly support the notion that there is an apparent bias of class II MHC-restricted T cells towards the recognition of structural and membrane-associated non-structural Kunjin virus proteins. This makes good biological sense for 2 reasons. Firstly, antigen-activated Th cells could participate in protective immunity by enhancing the production by B cells (through lymphokine secretion), of virus-neutralizing antibodies which must be directed against surface epitopes of viral structural proteins. Secondly, cell membrane-associated non-structural proteins could be the target for complement-dependent lysis of virus-infected cells. The protective role of such antibodies has been shown in experimentally-infected mice and in monkeys with yellow fever (Schlesinger *et al.*, 1985, 1986) and dengue viruses (Zhang *et al.*, 1988).

Apparent bias of class II MHC-restricted T cells for the recognition of structural and membrane-associated, non-structural protein can be explained on the basis of the relative availability of various virus-specified proteins for T cell recognition. Since the membrane-directed (C, PrM, E & NS1) proteins follow a pathway through ER and Golgi, it can be proposed that these proteins are less likely to be degraded by intracellular proteases, whereas the chance of cytosolic non-structural proteins being degraded by cellular enzymes are likely to be greater. Even after the lysis of infected cells as a result of either direct cytopathic effect of virus or Tc and NK cell-mediated lysis, the relative availability of virus-specified, membrane-associated proteins in their native state for antigen-uptake by various APC may be greater than those from non-structural proteins. It may be that during the initial phase of the immune response, class II^+ macrophages and DC present all potential epitopes to resting T cells. However, in a later phase of the immune response, when antigen concentration is low, antibody-dependent uptake of antigen may be dominant. Thus, if antibody responses are biased towards membrane-associated proteins, these proteins may be the dominant source of epitopes for $CD4^+$ T cells. The uptake of an antigen via clonally-distributed sIg present on antigen-specific B cells provides an efficient way for the intracellular accumulation of large quantities of epitope-carrying fragments (Howard, 1985) and specific B cells would be present in ever-increasing numbers during development of the immune response. Therefore it can be proposed that these APC will select only those T cell clones which reciprocally activate them (by secreting

lymphokines) to fulfil their functional commitment, i.e. differentiation into antibody secreting cells.

In addition, the recognition of 1022 by T cells implies that certain virus-encoded cytosolic non-structural proteins released by various mechanisms discussed above, are also available for processing by APC in the class II-restricted pathway. The relative accessibility of such antigens to different classes of APC such as macrophages, DC and B cells would depend upon sites of virus growth and the stage of pathogenesis of infection. There may be interaction between different APC as in the example where B cells take up antigens from follicular dendritic cells through an interactive pathway of antigen-presentation (Tew *et al.*, 1989).

A point of some interest was the observation that regardless of haplotype, the responses to 1024, which encompasses 1023 and 1022, were lower than the responses to 1022. Since optimal dilutions of lysates were used in the proliferation assays, these findings cannot be explained on the basis that 1024-pulsed macrophages expressed sub-or hyper-optimal concentrations of ligand, resulting in suppression (see following Chapter). These results may be explained as follows. Firstly, given that protein degradation is a function of its structure (Tanford, 1968; Allen, 1987), epitope-relevant fragments within 1024 might have been cleaved in a different way than those within 1022. Further constraints on the processing and presentation of these fragments include, 1) the residues distant from the minimal peptide epitope may affect processing by promoting or inhibiting unfolding or cleavage of fragments (Shastri *et al.*, 1986), and, 2) competition between

peptides for association with class II molecules may affect the quantity of a given epitope on the APC surface (Buus *et al.*, 1987). Thus, processed antigenic fragments derived from the cleavage of polyprotein of the replicating Kunjin virus following priming might differ qualitatively or quantitatively from those displayed by 1024-pulsed macrophages; whereas 1022, which encompasses mostly NS5, might be processed in the same way as during natural Kunjin infection. Disparity in binding of processed fragments derived from natural processing and corresponding synthetic peptides has been shown in the case of equine myoglobin (Brett *et al.*, 1988). Taken together, these arguments support the notion that differences in processing of 1024 and 1022 is the probable cause for lower responses to 1024.

In the initial experiments, when the responders and APC were co-cultured at a fixed ratio it was observed that within a given haplotype, both macrophages and B cells induced inconsistent patterns of responses from immune T cells. For example, WNV-immune CBA T cells responded more strongly to WNV-antigen-pulsed macrophages than WNV-antigen pulsed B cells (Table 4.2), but in the case of recombinant vaccinia virus-derived Kunjin antigens (Table 4.3, responses to 1031), Kunjin-immune CBA T cells responded as strongly to 1031 presented by B cells as by macrophages. Similarly, WNV-specific proliferative responses induced by B/b macrophages were significantly lower than those induced by B/b B cell APC (Table 4.2), but in the case of recombinant vaccinia virus-derived Kunjin antigens (Table 4.4), B/b macrophages were more efficient stimulators than B cells. Since the magnitude of T cell proliferation is a function of both antigen concentration and available MHC molecules (Matis *et*

al., 1983), then under the conditions of fixed antigen concentrations (WNV or recombinant vaccinia virus-derived Kunjin peptides), the observed variability of T cell responses could be attributed to inter-experimental variation in levels of MHC class II antigens on both types of APC and levels of antigen-specific B cells in primed spleen cell populations. It can be concluded from the results obtained from Ia^k-specific proliferative T cell responses that high stimulator cell numbers adversely affected the proliferative responses (see the following Chapter). Therefore varying numbers of macrophage APC were used to evaluate the MHC Ir gene effects with a view to provide a range of concentrations of available MHC molecules (complexed with WNV or Kunjin peptides) for inducing optimal responses from the majority of the clones within the polyclonal T cell populations.

With WNV antigen the T cells of H-2^b and H-2^d haplotypes more realibly gave good responses than H-2^k and H-2^q haplotypes (Fig 4.2). In the case of recombinant vaccinia virus-derived Kunjin antigens, responders of the H-2^b and H-2^d haplotypes predominantly recognized 1031 and 1022, whereas their responses to 1023 and 1024 were weak (Figs 4.4 and 4.5). In the H-2^k haplotype, both CBA and BR T cells predominantly recognized 1022 while responses to 1031 were weak (Fig. 4.3). The T cells from H-2^k, H-2^b and H-2^d haplotypes responded to Kunjin antigens in decreasing order of strength. Why this order differs from responses with WNV is not clear. Analysis of this question would require an investigation of cross-reactivity between WNV and Kunjin and ultimately identification of the peptides relevant to $CD4^+$ T cell epitopes.

The influence of Ir genes on these responses could be explained as follows, 1) variability in the MHC-binding affinity of processed antigenic peptides together with the competing self peptides may affect the concentration of MHC-peptide complexes displayed on the surface of APC for T cell recognition, or, 2) since the TcR usage for the recognition of a particular MHC-peptide combination is influenced both positively and negatively by thymic MHC molecules, it can be proposed that certain MHC molecules may simply be better able to interact with larger numbers of TcRs than others resulting in the selection of T cell clones either with high affinity or high precursor frequency.

Although, B cells of all haplotypes tested presented 1031 and 1022, their efficiency did not necessarily correspond to that of macrophages from the same mouse strains. These differences in the antigen-presenting function of macrophages and B cells could due to, 1) differences in antigen uptake and proteolytic mechanisms involved in antigen processing (Jensen, 1988; Harding and Unanue, 1988), and, 2) disparity in the generation of Ia-peptide complexes due to glycosylation differences in the α-chain of Ia from these two APC (Cullen *et al.*, 1981).

With regard to background genes, CBA macrophages, at an optimal S:R ratio of 1, induced higher responses from CBA T cells than BR T cells (Fig.4.2). Furthermore, BR macrophages did not stimulate the responses of BR T cells, even at optimal S:R ratios, as efficiently as CBA macrophages (Table 4.2 and Fig.4.2). However, BR B cells did not seem deficient in their APC function (Table 4.2). Taken together, these observations suggest that low responses of BR T cells *in vitro* could be due to poor ability of BR macrophages to present antigens for T cell priming *in vivo*. In

gerneral, under optimal stimulatory conditions *in vitro*, macrophages from B/b, B/c and CBA were more efficient stimulators of H-2-matched T cells than B6, D2 and BR macrophages. The reason for apparent low efficacy of macrophages from the C57BL background was that they caused suppression of Th cells when used at the high stimulator cell numbers which gave optimal responses with macrophages from other strains (see Chapter 5). In this case it may be possible that non-MHC genes may influence certain features of antigen processing and presentation, or qualitative and/or quantitative factors involved in adhesion between APC and T cells (Bierer *et al.*, 1989).

Summary

The present Chapter analyses the influence of MHC class II (Ir) genes on MHC class II-restricted T cell responses to West Nile and recombinant vaccinia virus-derived Kunjin antigens and identifies the immunodominant antigens encoded by the latter. WNV-specific *in vitro* T cell responses from H-2^b and H-2^d mice were higher than those from H-2^k and H-2^q haplotypes. Of the recombinant vaccinia virus-derived Kunjin antigen preparations tested *in vitro*, Kunjin-immune T cells of H-2^b and H-2^d haplotypes predominantly recognized structural (prM/M, C, E) and membrane-associated non-structural proteins (NS1) encoded by VKV 1031 and showed weaker responses to cytosolic non-structural protein, NS5 (VKV 1022), whereas the responders of H-2^k haplotype strongly recognized the antigens encoded by VKV 1022 and VKV 1031.

Within a given inbred strain, B cells and macrophages differed in their ability to present recombinant vaccinia virus-derived Kunjin antigens, both in terms of magnitude of T cell responses induced and the particular Kunjin protein presented. The observation that T cells from different backgrounds varied in their requirement of macrophage APC numbers for maximum reactivity suggested that the concentration of class II MHC antigens and other molecules affecting APC-T cell interaction varied in mice with different genetic backgrounds.

The finding that regardless of MHC haplotype, the responses to 1024 which encompasses 1023 and 1022, were lower than 1022, probably reflects the differences in the processing requirements of these two proteins.

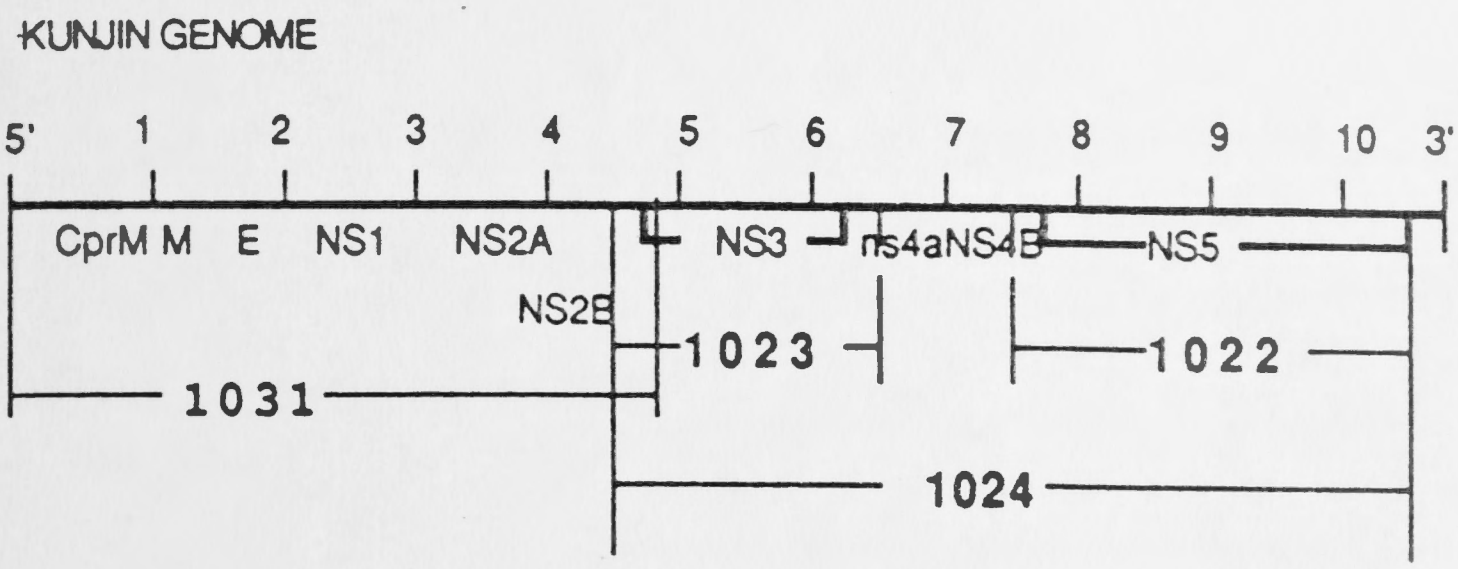


Fig. 4.1. Map of cDNA of the Kunjin genome (Coia *et al.*, 1988) showing the various segments encoded by Kunjin-vaccinia virus recombinants (Parish *et al.*, unpublished). VKV 1031 encodes, C, prM/M, E, NS1, NS2A, NS2B and a portion of NS3; VKV 1023 encodes for a portion of NS2B, all of NS3 and a portion of ns4a; VKV 1024 encodes, all of NS3-NS5 and a part of NS2B, while VKV 1022 encodes a portion of NS4B and all of NS5.

Fig. 4.2: *In vitro* responses of WNV-primed CD4+ T cells with varying numbers of macrophages (as APC) from different MHC haplotypes and backgrounds in the presence of a 1/4 dilution of WNV antigen stock (solid lines) or mock control (broken lines).

H-2^b haplotype:	Closed points:	BALB/b	responders
	Open points	C57BL/6	responders
	Squares	BALB/b	stimulators
	Circles	C57BL/6	stimulators
H-2^d haplotype:	Closed points	BALB/c	responders
	Open points	B10.D2	responders
	Squares	BALB/c	stimulators
	Circles	B10.D2	stimulators
H-2^k haplotype:	Closed points	CBA/H	responders
	Open points	B10.BR	responders
	Squares	CBA/H	stimulators
	Circles	B10.BR	stimulators
H-2^q haplotype:	Closed points	DBA/1J	responders
	Open points	B10.T(6R)	responders
	Squares	DBA/1J	stimulators
	Circles	B10.T(6R)	stimulators

Fig. 4.2

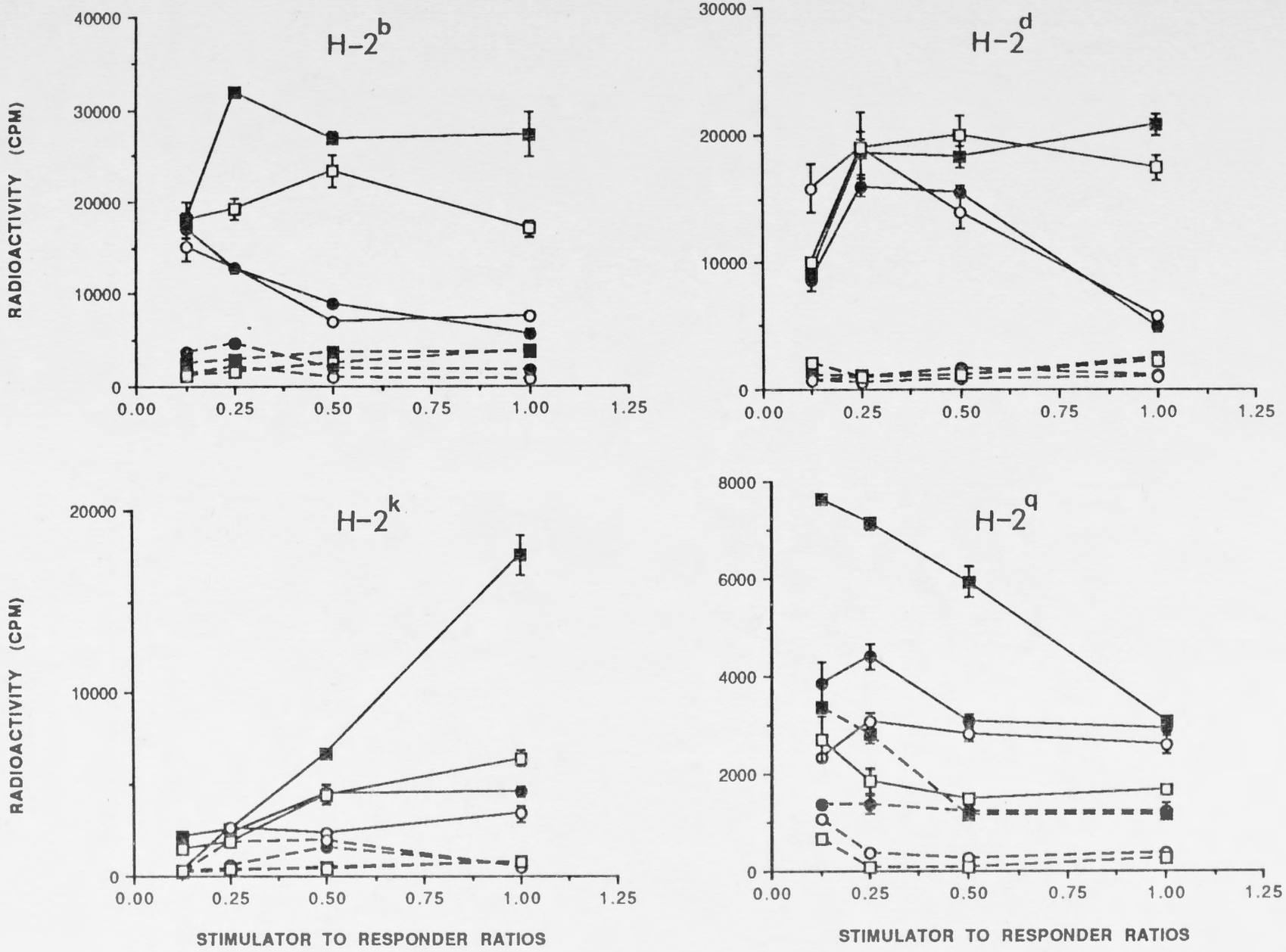

H-2b
H-2d
H-2k
H-2q
RADIOACTIVITY (CPM)
STIMULATOR TO RESPONDER RATIOS
0
10000
20000
30000
40000
2000
4000
6000
8000
0.00
0.25
0.50
0.75
1.00
1.25

Fig. 4.3:

In vitro responses of H-2^k-restricted Kunjin-primed $CD4^+$ T cells by varying numbers of macrophages pulsed with recombinant vaccinia virus-derived Kunjin antigens. CBA/H and B10.BR responders were stimulated by H-2-matched macrophages in the presence of a 1/4 dilution of lysates from CV1 cells infected with VVTK$^-$ (□), VKV 1024 (■), VKV 1023(●), VKV 1022 (○) or VKV 1031 (▲).

Fig. 4.3

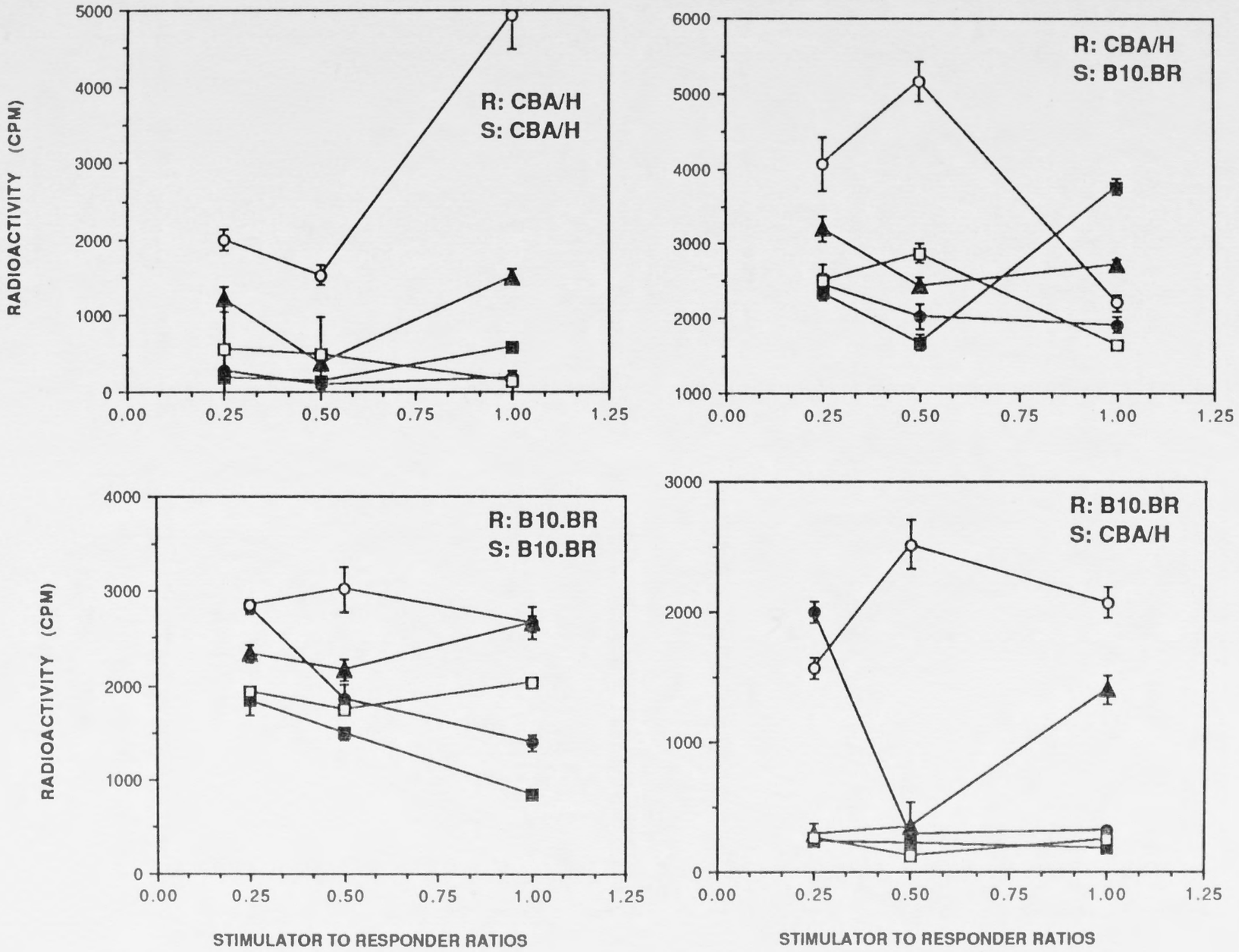

RADIOACTIVITY (CPM)
R: CBA/H
S: CBA/H
R: CBA/H
S: B10.BR
R: B10.BR
S: B10.BR
R: B10.BR
S: CBA/H
STIMULATOR TO RESPONDER RATIOS
STIMULATOR TO RESPONDER RATIOS

Fig. 4.4:

In vitro responses of H-2^b-restricted Kunjin-primed $CD4^+$ T cells by varying numbers of macrophages pulsed with recombinant vaccinia virus-derived Kunjin antigens. BALB/b and C57BL/6 responders were stimulated by H-2-matched macrophages in the presence of a 1/4 dilution of lysates from CV1 cells infected with $VVTK^-$ (□), VKV 1024 (■), VKV 1023 (●), VKV 1022 (○) or VKV 1031 (▲).

Fig. 4.4

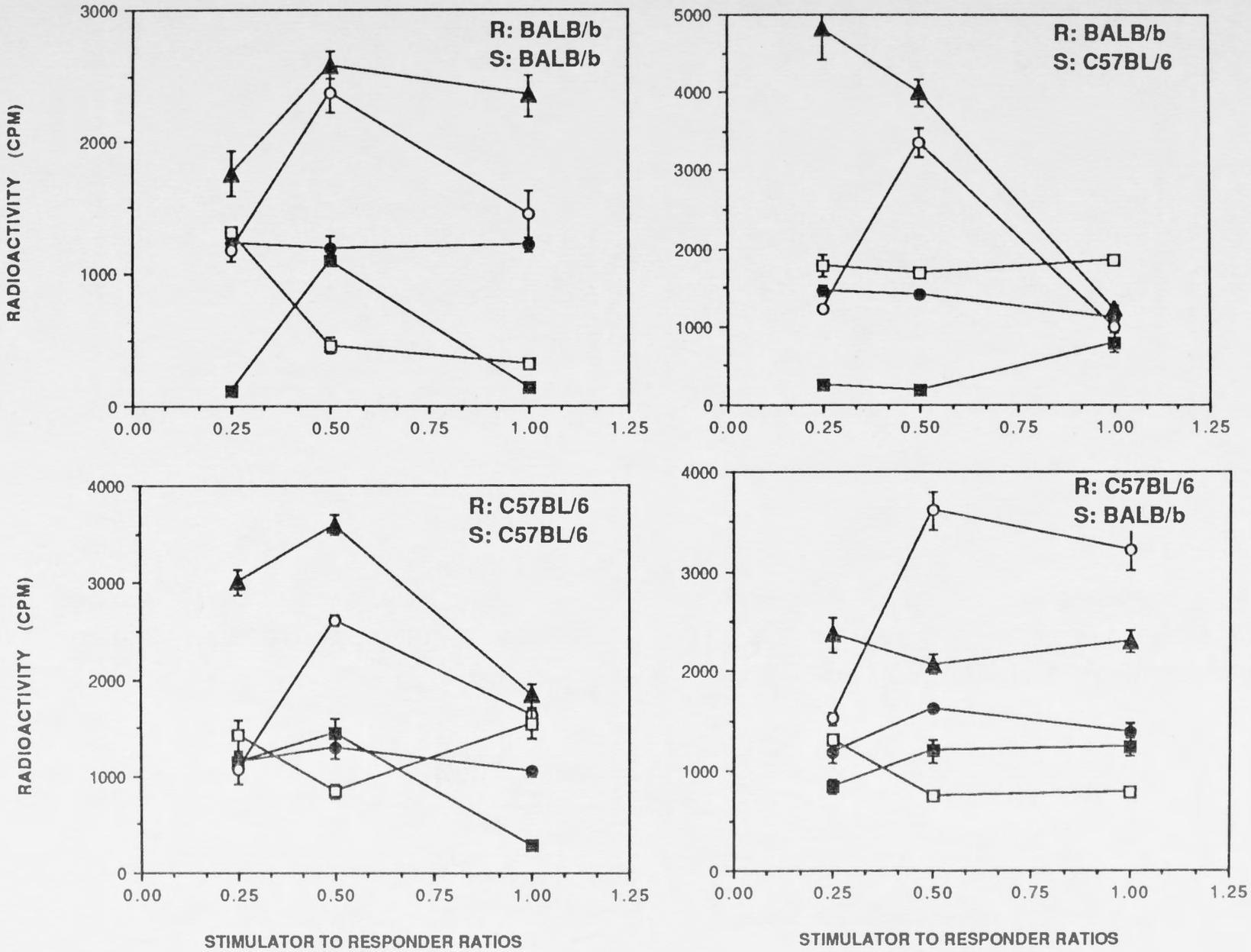

R: BALB/b
S: BALB/b
R: BALB/b
S: C57BL/6
R: C57BL/6
S: C57BL/6
R: C57BL/6
S: BALB/b
RADIOACTIVITY (CPM)
STIMULATOR TO RESPONDER RATIOS
0
1000
2000
3000
4000
5000
0.00
0.25
0.50
0.75
1.00
1.25

Fig. 4.5:

In vitro responses of H-2^d-restricted Kunjin-primed $CD4^+$ T cells by varying numbers of macrophages pulsed with recombinant vaccinia virus-derived Kunjin antigens. BALB/c and B10.D2 responders were stimulated by H-2-matched macrophages in the presence of a 1/4 dilution of lysates from CV1 cells infected with $VVTK^-$ (□), VKV 1024 (■), VKV 1023 (●), VKV 1022 (○) or VKV 1031 (▲).

Fig. 4.5

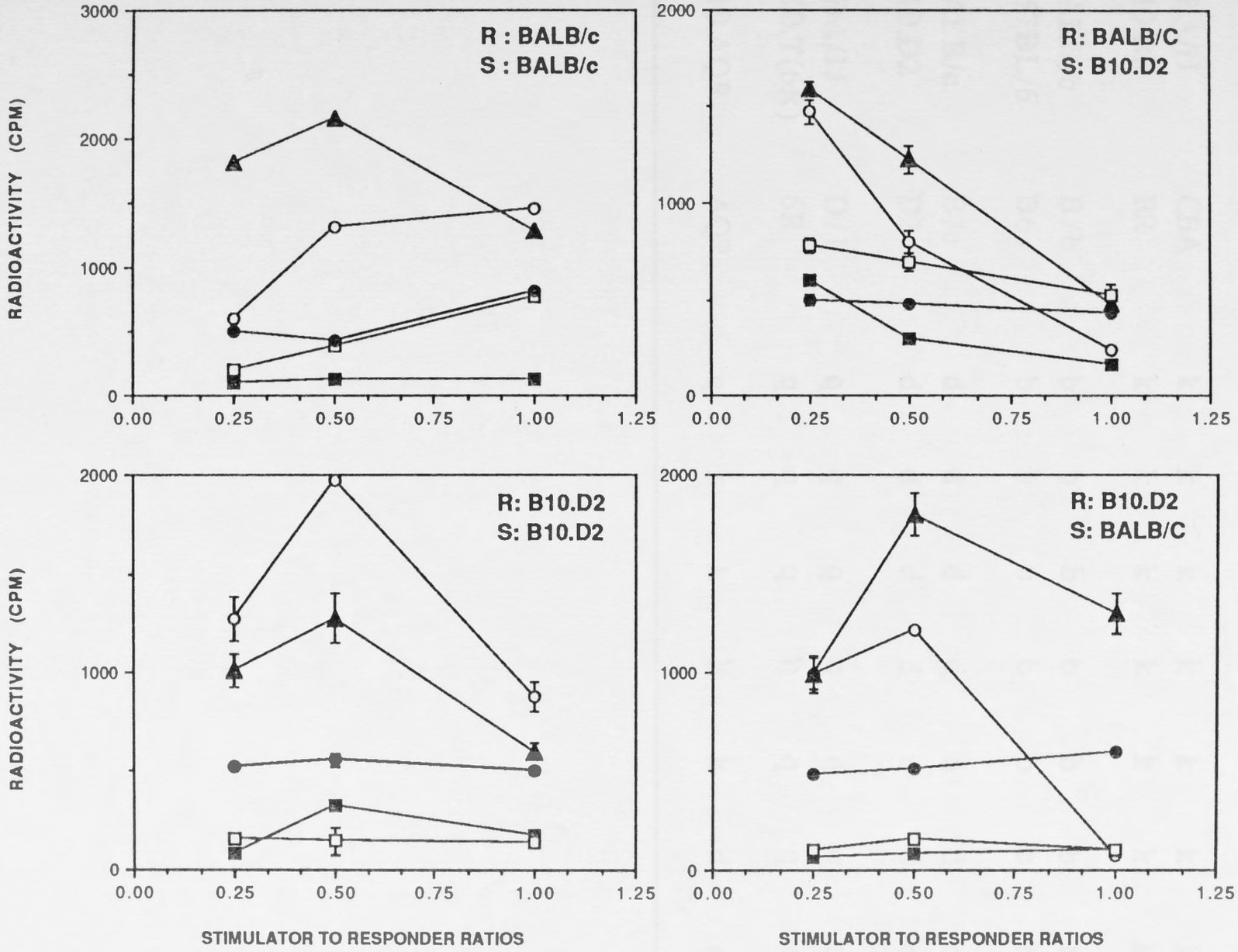

R : BALB/c
S : BALB/c
R: BALB/C
S: B10.D2
R: B10.D2
S: B10.D2
R: B10.D2
S: BALB/C
RADIOACTIVITY (CPM)
STIMULATOR TO RESPONDER RATIOS
0
1000
2000
3000
0.00
0.25
0.50
0.75
1.00
1.25

Table 4.1 : Strains of mice used and their H-2 haplotype.

Strain	Abbreviation	H-2 haplotype						
		K	Aα	Aβ	Eβ	Eα	S	D
CBA/H	CBA	k	k	k	k	k	k	k
B10.BR	BR	k	k	k	k	k	k	k
BALB/b	B/b	b	b	b	b	b	b	b
C57BL/6	B6	b	b	b	b	b	b	b
BALB/c	B/c	d	d	d	d	d	d	d
B10.D2	D2	d	d	d	d	d	d	d
DBA/1J	D/1	q	q	q	q	q	q	q
B10.T(6R)	6R	q	q	q	q	q	q	d
B10.AQR	AQR	q	k	k	k	k	d	d

Table 4.2:

[a] $2x10^5$ enriched $CD4^+$ T cells from mice primed with 10^6 pfu of WNV 14 days previously, were co-cultured with LM-macrophages and B cells (from 14 day WNV-primed mice) at a 1:1 ratio in the presence of a 1/4 dilution of WNV antigen stock (M + Ag or B + Ag) or mock control (M + Mo.Ag or B + Mo.Ag).

1,2; 1,5; 4,5; 7,11; 6,11 & 18,19	Statistically significant ($P<0.0005$),
2,3; 8,11; 9,11; 10,11; 7,12; 6,12; 11,13 & 6,13	Statistically significant ($P<0.0025$),
8,12 & 9,12	Statistically significant ($P<0.01$),
12,13; 14,15 & 16,17	Statistically significant ($P<0.05$),
6,7	Statistically insignificant ($P>0.1$),
6,8 & 7,9	Statistically insignificant ($P>0.25$),
2,4; 6,10 & 12,10	Statistically insignificant ($P>0.3$),
6,9	Statistically insignificant ($P>0.45$).

* Mean ^{3}H-TdR (cpm) uptake by at least triplicate cultures ± S.E.M.
The data are representative of one experiment.

Table 4.2 : *In vitro* stimulation of WNV-immune CD4+ T cells by macrophages and B cells from different backgrounds [a].

WNV immune	Stimulator	Responses of CD4+ T cells.			
		Macrophages as stimulators		14 day-WNV-primed B cells as stimulators	
CD4+ T cells	APC	M + Ag	M + Mo.Ag	B + Ag	B + Mo.Ag
CBA	CBA	*59,033 ± 3336 [1]	1854 ± 231	4376 ± 59	570 ± 29
CBA	BR	11,247 ± 957 [2]	1066 ± 102	4408 ± 477 [3]	1307 ± 56
BR	BR	3417 ± 283 [4]	448 ± 56	1677 ± 77	468 ± 45
BR	CBA	12,011 ± 1152 [5]	825 ± 62	1522 ± 106	526 ± 53
B6	B6	32,036 ± 2491 [6]	1022 ± 280	27,569 ± 2032 [7]	1226 ± 101
B6	B/b	7546 ± 442 [11]	1565 ± 8	28,493 ± 4183 [8]	1877 ± 250
B/b	B/b	10,252 ± 3326 [12]	2176 ± 84	29,360 ± 4123 [9]	2991 ± 173
B/b	B6	19,741 ± 2624 [13]	1519 ± 69	34,403 ± 4731 [10]	5822 ± 548
D/1	D/1	6509 ± 574 [14]	3654 ± 67	9519 ± 1031 [15]	842 ± 114
D/1	6R	1457 ± 98 [16]	307 ± 23	3089 ± 827 [17]	1236 ± 105
6R	6R	494 ± 38	344 ± 93	5372 ± 1554	1556 ± 51
6R	D/1	3318 ± 164 [18]	2777 ± 99	7569 ± 557 [19]	878 ± 43

Table 4.3:

[c] $2x10^5$ enriched $CD4^+$ T cells from 14 day Kunjin-primed mice were co-cultured with macrophages (a) or B cells (b) at a 1:1 ratio, in the presence of CV1 cell lysates from $VVTK^-$ control or Kunjin-VV-infected cells at a 1/4 dilution.

2,3 & 4,5	Statistically significant ($P<0.0025$),
6,7	Statistically significant ($P<0.01$),
1,3; 8,9 & 9,10	Statistically insignificant ($P>0.05$),
8,10	Statistically insignificant ($P>0.4$),
9,10	Statistically insignificant ($P>0.05$),
12,7	Statistically significant ($P<0.0005$),
13,14	Statistically significant ($P<0.05$).
*	As for Table 4.2.

Table 4.3 : H-2^k restricted *in vitro* proliferative responses of Kunjin-immune T cells to recombinant vaccinia virus-derived Kunjin antigens presented by macrophages and B cells [c].

Kunjin immune CD 4^+ T cells	APC	Responses to Kunjin antigens				Responses to
		VKV 1024	VKV 1023	VKV 1022	VKV 1031	VVTK$^-$
a, Macrophages as APC.						
BR	BR	*264 ± 40	538 ± 45	1235 ± 59 [1]	2699 ± 259 [2]	1009 ± 98 [3]
BR	CBA	240 ± 15	551 ± 29	1925 ± 264 [4]	4017 ± 171	1014 ± 58 [5]
CBA	CBA	267 ± 20	529 ± 31	1872 ± 225 [6]	3732 ± 22 [12]	1118 ± 45 [7]
CBA	BR	260 ± 9	828 ± 52	2381 ± 63 [8]	3069 ± 361 [9]	2403 ± 46 [10]
b, B cells as APC.						
BR	BR	*201 ± 10	379 ± 9	1050 ± 78	1140 ± 127	257 ± 9
BR	CBA	189 ± 7	509 ± 55	1128 ± 96	2539 ± 222	342 ± 27
CBA	CBA	233 ± 9	346 ± 8	1020 ± 67	4795 ± 309	273 ± 39
CBA	BR	205 ± 14	409 ± 14	530 ± 39 [13]	6532 ± 613	409 ± 30 [14]

Table 4.4:

[c] $2x10^5$ enriched $CD4^+$ T cells from 14 day Kunjin-primed mice were co-cultured with macrophages (a) or B cells (b) at a 1:1 ratio, in the presence of CV1 cell lysates from $VVTK^-$ control or Kunjin-VV-infected cells at a 1/4 dilution.

1,2	Statistically significant ($P<0.05$),
3,4	Statistically significant ($P<0.0025$),
3,9	Statistically insignificant ($P>0.15$),
5,6	Statistically significant ($P<0.0025$),
7,10 & 12,14	Statistically insignificant ($P>0.1$),
6,11 & 12,13	Statistically significant ($P<0.0005$).

* As for Table 4.2.

Table 4.4 : H-2^b restricted *in vitro* proliferative responses of Kunjin-immune T cells to recombinant vaccinia virus-derived Kunjin antigens presented by macrophages and B cells [c].

Kunjin immune CD4+ T cells	APC	Responses to Kunjin antigens				Responses to
		VKV 1024	VKV 1023	VKV 1022	VKV 1031	VVTK-
a, Macrophages as APC.						
B6	B6	*401 ± 36	759 ± 34	2227 ± 180 [1]	2905 ± 175 [2]	532 ± 23 [8]
B6	B/b	473 ± 74	1064 ± 57	1999 ± 225 [3]	5506 ± 850 [4]	1708 ± 33 [9]
B/b	B/b	967 ± 108	645 ± 11	2277 ± 118 [7]	5709 ± 510	1901 ± 20 [10]
B/b	B6	2081 ± 103	579 ± 71	1852 ± 20 [5]	2805 ± 85 [6]	1131 ± 14 [11]
b, B cells as APC.						
B6	B6	*1269 ± 95	927 ± 22	2118 ± 6	579 ± 114	350 ± 21
B6	B/b	1598 ± 107	808 ± 26	1716 ± 70	1214 ± 312	231 ± 31
B/b	B/b	1382 ± 140[12]	791 ± 33	2001 ± 26 [13]	3929 ± 109	1158 ± 101[14]
B/b	B6	1153 ± 124	1104 ± 184	1674 ± 315	1027 ± 86	446 ± 98

Table 4.5:

[c] $2x10^5$ enriched $CD4^+$ T cells from 14 day primed mice were co-cultured with macrophages (a) or B cells (b) at a 1:1 ratio, in the presence of CV1 cell lysates from $VVTK^-$ control or Kunjin-VV-infected cells at a 1/4 dilution.

1,2; 1,3; 2,4; 2,6; 3,4 & 3,7	Statistically significant ($P<0.0005$),
5,6	Statistically significant ($P<0.0125$).
*	As for Table 4.2.

Table 4.5 : H-2^d restricted *in vitro* proliferative responses of Kunjin-immune T cells to recombinant vaccinia virus-derived Kunjin antigens presented by macrophages and B cells [c].

Kunjin immune $CD4^+$ T cells	APC	Responses to Kunjin antigens (cpm)				Responses to
		VKV 1024	VKV 1023	VKV 1022	VKV 1031	$VVTK^-$ (cpm)
a, Macrophages as APC.						
D2	D2	*241 ± 18	717 ± 40	1297 ± 151	996 ± 65 [1]	1263 ± 103
D2	B/c	276 ± 14	990 ± 64	1678 ± 182 [5]	4545 ± 113 [2]	1095 ± 58 [6]
B/c	B/c	379 ± 68	812 ± 57	1182 ± 191	4796 ± 370 [3]	1401 ± 370 [7]
B/c	D2	289 ± 36	696 ± 19	1232 ± 38	939 ± 47 [4]	1295 ± 96
b, B cells as APC.						
D2	D2	*249 ± 24	570 ± 31	1563 ± 77	656 ± 17	410 ± 79
D2	B/c	252 ± 44	695 ± 48	1452 ± 19	1208 ± 34	441 ± 52
B/c	B/c	219 ± 14	638 ± 22	1515 ± 92	1414 ± 33	267 ± 22
B/c	D2	220 ± 11	686 ± 53	1688 ± 25	2715 ± 103	345 ± 35

Table 4.6 : Stimulation of anti-Ia^k T cells [a] by LM- or TH-macrophages from BR or CBA mice.

S:R ratios[b]	CBA Macrophages		BR Macrophages	
	Listeria-induced	Thioglycollate-induced[9]	*Listeria*-induced	Thioglycollate-induced[c]
1	*41,355 ± 466 [1]	23,592 ± 757 [3]	31,278 ± 455 [5]	11,188 ± 567 [7]
0.5	42,801 ± 2768	14,058 ± 1117	74,407 ± 1658	9443 ± 372
0.25	67,436 ± 3778	27,017 ± 1349	92,491± 1051	16,998 ± 960
0.125	67,090 ± 1647 [2]	43,426 ± 1888 [4]	78,520 ± 1960 [6]	46,377 ± 678 [8]

[a] Responses of alloreactive (anti-Ia^k) T cells upon stimulation with allogeneic peritoneal macrophages from *Listeria*-and thioglycollate-induced CBA & B10.BR mice. Macrophages were co-cultured at different concentrations with $2x10^5$ alloreactive T cells generated in primary culture.

[b] Stimulator to responder ratios.

[c] Peritoneal exudate cells were harvested 3 days after intraperitoneal injection of mice with 3 ml of 10% thioglycollate broth.

1,2; 3,4; 5,6 & 7,8 Statistically significant ($P<0.0005$).

* As for Table 4.2.

The data are representative of one experiment.

Table 4.7: Ability of Kunjin-VV recombinants to prime for secondary *in vitro* proliferative responses.

Responder $CD4^+$ T cells [a]	Responses to Kunjin antigens	
	M+Ag	M+Mo.Ag
VKV 1022	*6320 ± 190 [1]	3533 ± 303
VKV 1023	4797 ± 623 [2]	2888 ± 124
VKV 1024	5544 ± 443 [3]	3055 ± 444
VKV 1031	9003 ± 159 [4]	3397 ± 698
$VVTK^-$	1372 ± 115	1647 ± 131
Kunjin-immune [7]	6595 ± 253 [5]	2249 ± 278
Naive	3494 ± 141 [6]	2941 ± 244

[a] $2x10^5$ $CD4^+$ T cells from the naive or primed (Kunjin-VV recombinants or Kunjin-strain MRM 16) were co-cultured with $4x10^5$ LM-macrophage APC in the presence of a 1/4 dilution of Vero cell-derived Kunjin antigen stock (M+Ag) or mock control (M+Mo.Ag).
Stimulator to responder ratio = 0.25.

[4,5 & 5,6] Statistically significant ($P<0.0005$); [1,5] Statistically insignificant ($P>0.2$);
[2,5] Statistically significant ($P< 0.025$); [3,5] Statistically significant ($P<0.05$).
[7] $CD4^+$ T cells from the mice primed with Kunjin-strain MRM 16.
* As for Table 4.2.
The data are representative of one experiment.

CHAPTER 5

EFFECT OF HIGH LIGAND CONCENTRATION ON WEST NILE VIRUS-SPECIFIC T CELL PROLIFERATION

Introduction

Activation of class II MHC-restricted CD4+ T cells requires recognition of antigenic peptides bound to class II molecules present on the surface of APC. In optimizing the numbers of APC reported in Chapter 2, 3 and 4, it was observed that high APC numbers and high WNV antigen concentrations suppressed CD4+ T cell proliferative responses. Using cloned T cells, Matis *et al* (1983) have demonstrated that the magnitude of antigen-induced proliferation is a function of the product of antigen concentration and number of Ia molecules on APC. Based on these observations, the suppression of proliferation at high antigen concentration was thought to be due to excess number of TcR occupancy by macrophage-bound complex ligands. In the present experiments the relative contribution of WNV antigen concentration and MHC class II antigen availability to the suppression of T cell proliferation was investigated.

Materials and Methods

Mice:

C57BL/6, BALB/b (H-2^b), CBA/H (H-2^k), B10.AQR (K^q I^k D^d) and B10.T(6R) (K^q I^q D^d) strains of mice bred under specific pathogen-free conditions at the JCSMR were used at 8 to 10 weeks of age.

Virus:

Mice were primed with 10^6 pfu of WNV as described in Chapter 2.

Antibodies:

As described in Chapter 2. Monoclonal anti-I-$A^{b,d,q}$ (M_5/114.15, IgG2b) and anti-I-A^k (11.52.1, IgG2b) antibodies were used as neat culture supernatant. WNV-specific IgG was purified from WNV-immune ascitic fluid, as described in Chapter 3. Hybridoma line secreting anti-D^b antibodies (HB 36) was obtained from the American Type Culture Collection (Rockville, MD, USA).

Vero-cell derived WNV antigen:

As described in Chapter 2. This WNV antigen preparation was used at a 1/4 dilution in all the experiments unless otherwise stated.

Antigen presenting cells:

Four different types of APC used in the present study were: 1) *Listeria*-induced peritoneal macrophages (LM macrophages), obtained 3 days after intraperitoneal injection of live *Listeria monocytogenes*, 2) B^{++++} cells obtained from the mice hyper-immunized with WNV, 3) B_{14} cells obtained from 14 day WNV-primed mice, and, 4) B_0 cells from naive mice (described in Chapter 3).

B cells were irradiated with 1000 rads for use as APC.

In experiments disigned to test the effect of sustained MHC class II expression on LM and TH macrophages, the putative levels of these antigens were maintained on the macrophage APC *in vitro* cultures (Beller and Unanue, 1981) as follows. LM or Th PEC were plated directly into 96 well round-bottomed tissue culture plates. Non-adherent cells were removed after 2 h incubation at 37^0 C in a humidified atmosphere of 5% CO_2 in air and remaining adherent cells were incubated with hkLM (10^6 bacteria per well) in 100 µl DMEM supplemented with 1% NMS. After 1 h, adherent

macrophage populations were vigorously washed three times with DMEM to remove hkLM, before co-culturing them with responder T cells. In T cell proliferation assays, macrophage cultures used as APC were supplemented with $2x10^{-5}$ M of indomethacin (7 μg/ml of culture) unless otherwise stated.

Generation of anti-Ia^k-specific T cells:

A bulk culture of primary anti-Ia^k T cells was prepared as described in Chapter 3.

Preparation of Fab fragments from class II antibodies:

Proteolysis of anti-Ia^b and anti-Ia^k (IgG2b) antibodies with the enzyme papain (Sigma, St. Louis, MO, USA) and the subsequent purification of monovalent Fab fragments and Fc fragments, using protein A Sepharose, was done as described by Mage (1980).

Proliferation assay:

Proliferative responses of immune $CD4^+$ T cells to WNV antigens were tested using modified assay as described in Chapter 2.

Statistical analysis:

As for Chapter 2.

Results

The influence of APC numbers on the proliferation of WNV-specific $CD4^+$ T cells:

B6 and B/b macrophages, pulsed with WNV antigens, were used as APC for B6 WNV-immune $CD4^+$ T cells at 5 different S:R ratios of 1.0 down to 0.031 in 2-fold steps. With B6 APC, T cell proliferation was maximal at S:R ratios of 0.25 to 0.125 and declined progressively both above and below the optimal

S:R ratios (Table 5.1). With B/b APC, strong proliferation occurred over a broad range of S:R ratio from 0.5 to 0.062, but at both ends of the range (1.0 and 0.031) there were precipitous falls in response. While responses to WNV antigen were well above mock antigen controls throughout the range of S:R ratios tested, the difference in antigen-induced responses between the worst and best ratios varied almost 6-fold with B6 APC and almost 10-fold with B/b APC.

B cells obtained from three different sources were evaluated for their APC function for the stimulation of WNV-immune $CD4^+$ T cells at 4 different S:R ratios ranging from 2.0 to 0.25 in 2-fold steps. In order to overcome the problem of suppression of T cell proliferation at a 1/4 dilution of WNV antigen (Chapter 3, Fig. 3.1), more dilute (1/16) WNV antigen was used to pulse these APC. The responses induced by B_0 cells increased with increasing numbers of B_0 cells used (Table 5.2). No suppression was observed at high S:R ratios. In contrast, B_{14} and B^{++++} cells showed an inverse dose response with suppression of T cell proliferation at higher B cell numbers. This suppression was most pronounced with B^{++++} APC at a S:R ratio of 2. These results suggest that hyper-optimal concentrations of ligand displayed by antigen-specific B cells within the B_{14} and B^{++++} cell populations caused the suppression of T cell proliferation at high S:R ratios. This consideration is based on the fact that uptake of an antigen via sIg on B cells results in the intracellular accumulation of high concentrations of potential epitope-carrying fragments (Howard, 1985).

These data confirm earlier findings (Chapters 2 and 4) that high numbers of APC adversely affected WNV-specific T cell proliferation.

Effect of sustained MHC class II expression on LM macrophages upon their capacity to stimulate and suppress alloreactive T cell proliferation:

To further investigate whether the inhibition of T cell proliferation was due to supra-optimal concentrations of ligand expressed by APC, the putative high levels of MHC class II antigens on LM macrophage APC were maintained by giving a phagocytic stimulus in the form of hkLM for 1 h, before the addition of immune T cells (Beller and Unanue, 1981). TH macrophages, which do not express high levels of Ia antigens, were used as a control for Ia^+ LM macrophages. The responses of alloreactive, Ia^k-specific T cells to these macrophage APC at various S:R ratios are shown in Table 5.3.

LM, LM plus hkLM and TH plus hkLM macrophages all showed inverse dose responses with the highest T cell proliferation occurring at a S:R ratio of 0.25 and profound suppression of responses at a S:R ratios of 1 and/or 2. In contrast, TH macrophages showed a direct dose response, with increasing T cell proliferation as APC numbers increased. These results are consistent with the idea that high ligand (class II MHC) expression on APC surfaces stimulates $CD4^+$ T cell proliferation at optimal APC numbers, but causes suppression at supra-optimal APC numbers.

The effect of indomethacin on the suppression of anti-WNV T cell proliferation caused by macrophage APC: Although the macrophage cultures were routinely supplemented with indomethacin at a concentration of 7 μg/ml (Mizel, 1981), which has been shown to inhibit synthesis of PG (Vane, 1971), it could be argued that at high S:R ratios, the levels of added indomethacin might be inadequate to prevent formation of PG and result in the suppression of T cell proliferation. Therefore, cultures in which anti-WNV T cells were suppressed by LM macrophage APC at a S:R ratio of 1 were supplemented with varying concentrations of indomethacin ranging from 7 to 28 μg/ml. Data presented in Table 5.4 indicate that addition of indomethacin at a concentrations up to 28 μg/ml did not completely remove the suppression, suggesting that it was not mediated by PG.

Effect of varying WNV-antigen concentration on the optimal and suppressive responses induced by APC: In order to examine the contribution of WNV antigen concentration to suppression, WNV-specific T cell responses were measured over a range of WNV antigen dilutions. LM macrophages and B_{14} cells were used as APC at 2 different S:R ratios. With LM macrophage APC at a S:R ratio of 1, an inverse WNV-antigen dose response was seen (Table 5.5), i.e., progressively decreasing T cell proliferation as WNV antigen concentration was increased. This could be not be attributed to some simple non-specific adverse effect of the WNV antigen preparation, because with LM macrophage APC at a S:R ratio of 0.25 a direct antigen dose response was obtained. There was progressively increasing T cell proliferation as WNV antigen

concentration was increased. With the B_{14} cell population used in this experiment, only direct antigen dose responses occurred at S:R ratios of 0.25 and 2, the only hint of suppression being seen at a 1/4 dilution of WNV antigen at a S:R ratio of 2. These data are consistent with the idea that high ligand (class II MHC complexed with WNV peptides) concentrations were responsible for suppression of anti-WNV $CD4^+$ T cell proliferation.

Specificity of the suppressive effects of APC on WNV-specific T cell proliferation:

To examine the specificity of this suppression by APC, the effect of adding WNV-specific IgG antibodies on the antigen-presenting function of B^{++++} cells was tested. In this experiment, using a S:R ratio of 2, cultures were supplemented with varying concentrations of anti-WNV IgG antibodies, the objective being to reduce uptake of WNV antigens via sIg on WNV-specific B cells (see Chapter 3). A marked improvement in the proliferative responses, which were even higher than those obtained at the optimum S:R ratio of 0.25, was seen following the addition of these antibodies at 2 and 4 mg/ml (Table 5.6). However, the apparent reversal of suppression also occurred in the presence of anti-influenza antibodies present at 1 mg/ml, suggesting the possibility that non-specific binding of IgG to either the B cell APC or T cells might counter putative high ligand-induced suppression.

The specificity of suppression was also investigated using anti-class II MHC antibodies. The responses of WNV-immune T cells from C57BL/6 mice to WNV antigens were investigated, using B^{++++} cells as APC, in the presence of varying dilutions of anti-Ia^b (IgG2b) and anti-Ia^k (class matched, IgG2b) antibodies.

In this approach, the objective was to block access of T cell receptors to class II MHC-WNV peptide complexes on APC surfaces. Addition of anti-Ia^b antibodies at dilutions of 1/200, resulted in over 3-fold improvement in responses (Table 5.7). Although anti-Ia^k antibodies (1/200) resulted in a greater than 2-fold improvement in response, it was significantly lower than those obtained with anti-Ia^b antibodies used at a similar dilution. The use of all dilutions of both antibodies gave some improvement in responses, perhaps for non-specific reasons as suggested above.

In a similar experiment using macrophage APC from C57BL/6 mice, the addition of anti-Ia^b antibodies at a dilution of 1/1500 resulted in a significant increase in T cell in response, in cultures with a S:R ratio of 2 (Table 5.8). Anti-Ia^b at 1/30 "blocked" T cell response significantly. There was no improvement in the T cell responses in the presence of anti-D^b antibodies.

The possibility that non-specific improvement in C57BL/6 T cell responses in presence of anti-Ia^k antibodies (Table 5.7) was due to steric hindrance of T cell-APC interaction caused by IgG bound to the interacting cells via the Fc portion of the molecule was tested. Responder T cells were co-cultured with LM-macrophages at a S:R ratio of 1 in presence of WNV antigen, Fab fragments derived from anti-Ia^b and anti-Ia^k antibodies and Fc fragments. As shown in Table 5.9, addition of Ia^b-specific Fab at a final concentration of 1 μg/ml showed a significant improvement in the responses, comparable with those obtained at a S:R ratio of 0.5, whereas Ia^k-specific Fab and Fc fragments had no effect.

Discussion

In Chapters 2, 3 and 4 it was demonstrated that the presence of large numbers of APC ($2x10^5$ per well) such as B_{14}, B^{++++} and LM macrophages caused a decrease in the proliferation of WNV-specific T cells. In the light of a similar observation that high numbers of SDC caused low Tc cell responses in 5 day MLR cultures (Pereira, 1986), this phenomenon of high APC number-induced suppression of T cell proliferation was investigated in studies reported in this Chapter.

At the outset it seemed possible that the suppression mediated by high numbers of macrophages could have been due to PG released by activated macrophages. The results described in Table 5.4, indicate that even after supplementing the cultures with indomethacin at 4 times the usual concentration required for inhibiting the synthesis of PG (Mizel, 1981), no major improvement in the responses was observed. Therefore, it is unlikely that the suppression observed is mediated by PG. In addition, suppression was also observed with B cells which are not known to produce PG. Furthermore, the observations that, 1) TH macrophages induced maximum proliferative responses from alloreactive, Ia^k-specific T cells at high S:R ratio (Table 5.3), 2) a progressive increase in the responses of WNV-specific T cells occurred in the presence of high macrophage numbers and decreasing WNV antigen concentrations (Table 5.5), and, 3) improvement in the responses of WNV-specific, H-2^b-restricted T cell responses occurred in the presence of high numbers of LM macrophage or B^{++++} cell APC and appropriate dilutions of anti-Ia^b antibodies (Tables 5.7 and 5.8); argue against the possibility

that metabolically active LM macrophages (or B^{++++} cells) caused the exhaustion of nutrients from the culture medium.

Finally, since the assays were performed using $CD4^+$ enriched T cell populations (i.e. depleted of $CD8^+$ T cells) it is unlikely that $CD8^+$ suppressor T cell activity could account for this phenomenon.

In light of the fact that the ligand recognized by T cells is a complex of an antigenic peptide bound to MHC, it was speculated that at high S:R ratios, the concentration of ligand may adversely affect T cell responses. Therefore, to test the above prediction, both components of the ligand were experimentally varied, viz. a) MHC class II concentration, and, b) nominal antigen (WNV antigen).

Firstly, the responses of MHC class II-specific (Ia^k) alloreactive T cells recognizing class II MHC antigens on LM and TH macrophages was studied. The levels of MHC antigens, which generally decay *in vitro* (Beller and Unanue, 1981) were maintained (or possibly up-regulated) by giving a phagocytic stimulus in the form of hkLM (Beller and Unanue, 1981). Enhanced suppression of Ia^k-specific T cell proliferation observed in the presence of LM + hkLM at a S:R ratio of 1, together with the observation that TH + hkLM also showed inverse dose responses comparable with those obtained with LM or LM + hkLM APC, clearly indicated that increased levels of MHC class II antigens increased suppression. The apparent reason for TH macrophages inducing direct dose responses is that low levels of Ia antigens on these macrophages (Beller *et al.*, 1980) are insufficient to cause suppression of T cell proliferation. However, in the presence of a phagocytic stimulus with hkLM there could

be up-regulation of the levels of MHC class II antigens, which resulted in the suppression of T cell proliferation observed. Consistent with these results are the findings reported earlier in Chapter 4 (Table 4.6), that the responses of alloreactive, Ia^k-specific T cells to both LM and TH macrophages from CBA and B10.BR mice showed an inverse dose response, although the magnitude of response with the latter APC type was lower.

Conversely, the MHC concentration on the APC was reduced by introducing anti-Ia^b antibodies into C57BL/6 (H-2^b) cell cultures. Data in Tables 5.7 and 5.8 shows that the addition of specific anti-Ia antibodies effectively reversed the suppression of WNV-specific $CD4^+$ T cell proliferation. These results also suggest that one of the reasons for the suppression of T cell proliferation was high levels of MHC antigens expressed on APC. A non-specific improvement in the proliferative T cell responses in presence of anti-Ia^k antibodies was possibly due to binding of these antibodies to FcRs present on B cells (Basten *et al.*, 1972) and/or activated T cells (Andersson *et al.*, 1981). Steric hindrance might have reduced the contact of TcR with the available ligand on APC, thereby reducing the amount of TcR-ligand interaction normally occurring. The use of anti-Ia^b Fab fragments significantly reversed the suppression of T cell proliferation, whereas Fab of anti-Ia^k had no effect, reinforcing the possibility that FcR binding of anti-Ia^k antibodies might have caused the previous non-specific improvement in the responses. It may be that steric hindrance caused by FcR-mediated binding of anti-Ia antibodies to APC and/or T cells was abolished when the monovalent Ia-specific Fab fragments were used. Small-sized Fc fragments on their own may not sterically hinder TcR-APC interaction despite

binding to FcRs on APC and/or T cells. In sum, these experiments indicate that high levels of MHC class II antigens were one of the contributing factors for the suppression of T cell proliferation observed at high APC numbers.

The effect of nominal antigen (WNV antigen) on high APC-induced T cell suppression was also investigated using two experimental approaches. Initially, WNV-specific T cell proliferation induced by high (supraoptimal) APC numbers (LM macrophages and B_{14} cells) in the presence of varying concentration of WNV antigen was evaluated. When the ligand concentration was maximum (at the lowest dilution of WNV antigen), proliferative responses were suppressed. However, as the concentration WNV antigen was progressively reduced, the T cell responses increased. Interestingly, the situation was quite different when the responses of T cells at lower (optimal) APC numbers were measured. Maximum T cell responses now occurred at the highest WNV antigen concentration (1/4 dilution) and reduction in the WNV antigen concentration resulted in sub-optimal responses.

The contribution of nominal antigen was also studied by using B^{++++} cells as APC. In Chapter 3, it was shown that sIg-mediated uptake of antigen is probably the major mechanism for antigen-uptake by these cells, as their APC function was inhibited by WNV-specific IgG antibodies. The possibility that high B^{++++} cell numbers causing the suppression of T cell proliferation was due to rapid uptake of antigens via sIg and concomitant expression of high ligand concentration was investigated. By adding anti-WNV antibodies, the antigen uptake via sIg was presumably reduced, resulting in a significant improvement in

the responses. However, increased responses were also detected when anti-inflenza IgG antibodies were used, raising the possibility of a non-specific effect of these antibodies dependent upon FcR binding as discussed above.

Taken together, the above findings support the notion that the concentrations of both MHC and the nominal antigen contribute toward the suppression of T cell proliferation observed at high APC numbers. A possible theoretical implication of this phenomenon to T cell tolerance will be discussed in Chapter 6. A practical consideration of this effect is that for *in vitro* analysis of T cell responses, it is imperative to optimize the variables that determine the ligand concentration, which may otherwise obscure the evaluation of antigen-specific responses.

Summary

In this Chapter, the capacity of large numbers of macrophage and hyper-immune B cell APC to suppress the proliferative T cell responses was investigated. It was observed that this suppression was not mediated by PG, as the use of indomethacin in cultures at 4 times the usual concentration did not reverse the suppression. This suppression was not due to the exhaustion of nutrients from the culture medium. Experiments were designed to evaluate the contribution of MHC and the nominal antigen in causing the suppression of T cell proliferation. LM and TH macrophages when incubated with hkLM *in vitro* to increase or maintain high MHC class II levels before the addition of alloreactive, Ia^k-specific T cells, showed inverse dose responses

with the highest T cell proliferation occurring at a S:R ratio of 0.25 and profound suppression at a S:R ratio of 1 or 2. However, TH macrophages alone which express low MHC class II levels, showed a direct dose response with increasing T cell proliferation as APC numbers increased. Addition of anti-Ia antibodies (or their Fab fragments) in the cultures at high APC numbers resulted in a significant improvement in the proliferative responses, indicating that high levels of MHC class II antigens contributed to suppression of T cell proliferation.

Similarly, the reversal of suppression of anti-WNV T cells caused by high numbers of LM macrophage and B_{14} cell APC was achieved by reducing the concentration of WNV antigen.

The observations support the notion that the suppression of T cell proliferation observed at high S:R ratios was due to supra-optimal concentrations of ligand on APC.

Table 5.1 : Influence of macrophage[1] APC number on the proliferative responses of WNV-immune T cells.

Responder $CD4^+$ T cells	Macrophage APC	S:R ratios[2]	Responses of immune T cells (cpm)	
			M + Ag	M + Mo.Ag
C57BL/6	C57BL/6	1.00	*7038 ± 867 [a]	2662 ± 56
		0.5	16,363 ± 811 [b]	1830 ± 249
		0.25	40,883 ± 2297[c]	3942 ± 409
		0.125	37,713 ± 1190	1420 ± 58
		0.062	26,443 ± 1952[e]	1136 ± 49
		0.031	17,408 ± 452 [f]	768 ± 30
C57BL/6	BALB/b	1.00	11,077 ± 674 [g]	2037 ± 286
		0.5	38,863 ± 286 [h]	4193 ± 450
		0.25	35,911 ± 309 [i]	5063 ± 433
		0.125	47,886 ± 4044	5144 ± 112
		0.062	51,397 ± 3629[k]	4020 ± 175
		0.031	5302 ± 416	3575 ± 42

1 LM macrophages from C57BL/6 and BALB/b mice were co-cultured at different concentrations with $2x10^5$ enriched $CD4^+$ T cells from 14-day WNV-primed C57BL/6 mice in the presence of a 1/4 dilution of WNV-antigen stock (M + Ag) or mock control (M + Mo.Ag).

2 Stimulator to responder ratios.

a,c;b,c;c,f & g,k Statistically significant ($P<0.0005$)

h,k & i,k Statistically significant ($P<0.0025$)

c,e Statistically significant ($P<0.005$)

* Mean ^{3}H-TdR uptake by at least triplicate cultures ± S.E.M.

The data are representative of one experiment.

Table 5.2 : Responses of WNV-immune T cells[a] to the varying numbers of B cell APC, pulsed with WNV-antigen.

B cell APC	S:R ratios[b]	Responses of immune CD4+ T cells (cpm)	
		B + Ag	B + Mo.Ag
[c] B_0	2	*8322 ± 616[1]	283 ± 79
	1	7792 ± 689	351 ± 46
	0.5	4791 ± 539	169 ± 32
	0.25	2300 ± 341[2]	136 ± 23
[c] B_{14}	2	2932 ± 176[3]	125 ± 12
	1	4491 ± 353	253 ± 46
	0.5	5773 ± 790	180 ± 44
	0.25	6157 ± 58[4]	137 ± 10
[c] B^{++++}	2	1170 ± 66[5]	64 ± 4
	1	5135 ± 569	90 ± 4
	0.5	4997 ± 502	155 ± 17
	0.25	6897 ± 973[6]	198 ± 8

[a] $2x10^5$ $CD4^+$ T cells from 14-day WNV-primed mice were co-cultured with $2x10^5$ irradiated B cells in the presence of a 1/16 dilution of WNV antigen stock (B + Ag) or mock control (B + Mo.Ag).

[b] As for Table 5.1.

[c] B_0, B_{14} & B^{++++} ---B cells from naive, 14-day-WNV-primed, and from the mice receiving weekly injections of WNV over a period of four weeks respectively.

1,5 & 3,5 Statistically significant ($P<0.0005$);

1,2; 1,3; 2,4; 3,4 & 5,6 Statistically significant ($P<0.0025$);

2,6 Statistically significant ($P<0.01$);

4.6 Statistically insignificant ($P>0.25$)

* As for Table 5.1

The data are representative of one experiment.

Table 5.3 : Stimulation of anti-Ia^k T cells by LM- and TH-macrophages[a] from CBA (H-2^k) mice.

S:R ratios [b]	LM-macrophages	TH-macrophages	LM-macrophages + hkLM	TH-macrophages + hkLM
2	ND	12,986 ± 1203[3]	ND	7099 ± 63[7]
1	*11,542 ± 2048[1]	3715 ± 1313	2203 ± 348[5]	8007 ± 1723
0.5	49,400 ± 4291	1650 ± 212	37,604 ± 3244	26,347 ± 1934
0.25	50,399 ± 7532[2]	2042 ± 214[4]	119,890 ± 4616[6]	52,802 ± 1478[8]

[a] LM and TH macrophages were cultured at different concentrations with or without heat-killed *Listeria* (hkLM, 10^6 bacteria) for 1 h before the addition of alloreactive (anti-Ia^k) T cells ($2x10^5$), generated in primary cultures.

[b] As for Table 5.1.

ND Not done.

[1,2 & 3,7] Statistically significant ($P<0.005$)

[3,4] Statistically significant ($P<0.0025$)

[4,8; 5,6 & 7,8] Statistically significant ($P<0.0005$)

* As for Table 5.1.

The data are representative of one of the two experiments performed.

Table 5.4 : An investigation of the capacity of indomethacin to reverse the suppressive effect of LM macrophages on WNV-specific T cell proliferative responses [a].

S:R ratios [b]	Responses of CD4+T cells		Concentration of added indomethacin µg/ml
	M + Ag	M + Mo.Ag	
1	*5400 ± 415	297 ± 21	7
0.5	10,244 ± 803	292 ± 18	7
0.25	25,272 ± 1920	848 ± 91	7
0.125	31,893 ± 3553[3]	465 ± 23	7
1	5875 ± 180[1]	399 ± 33	7
1	6070 ± 439	393 ± 24	14
1	6739 ± 493	498 ± 66	21
1	6984 ± 388[2]	297 ± 16	28

[a] Data from the upper panel shows a typical effect of high APC numbers on the proliferative responses of $2x10^5$ WNV-specific CD4+ T cells in the presence of a 1/4 dilution of WNV antigen stock (M + Ag) or mock control (M + Mo.Ag) & 7µg/ml of indomethacin. Data from the lower panel shows the effect of added indomethacin on WNV-specific T cell proliferation at a S:R ratio of 1.

[b] As for Table 5.1.

[1, 2,] Statistically significant ($P< 0.0125$).

[2,3] Statistically significant ($P< 0.0005$).

* As for Table 5.1.

The data are representative of one experiment.

Table 5.5 : Effect of varying antigen concentration on the antigen-presenting function of macrophages & B cells[a]

Dilution of antigen	Macrophage APC stimulator to responder ratios				B_{14} APC stimulator to responder ratios			
	1		0.25		2		0.25	
	M + Ag	M + Mo.Ag	M + Ag	M + Mo.Ag	B + Ag	B + Mo.Ag	B + Ag	B + Mo.Ag
1/4	* 754 ± 40	1480 ± 118	5245 ± 548	141 ± 23	5288 ± 355[1]	1335 ± 138	5269 ± 648	1233 ± 117
1/8	1187 ± 158	1519 ± 132	5062 ± 296	114 ± 4	6780 ± 206[2]	1863 ± 174	3954 ± 403	1141 ± 254
1/16	1170 ± 110	470 ± 46	4061 ± 226	173 ± 10	5355 ± 210	803 ± 94	3687 ± 549	724 ± 36
1/32	1766 ± 79	196 ± 35	4106 ± 470	186 ± 48	5403 ± 360	1724 ± 82	2436 ± 290	311 ± 7
1/64	2545 ± 84	171 ± 22	3563 ± 315	574 ± 3	3946 ± 104	1234 ± 34	1156 ± 87	782 ± 63
1/128	2736 ± 174	154 ± 7	1942 ± 174	400 ± 5	2292 ± 164	526 ± 40	1182 ± 103	357 ± 32

[a] $2x10^5$ enriched $CD4^+$ T cells from 14-day-WNV-primed mice were co-cultured with LM-macrophages or 14-day-WNV-primed B cells at the indicated ratios, in the presence of varying dilutions of WNV antigen stock (M + Ag or B + Ag) or mock control (M + Mo.Ag or B + Mo.Ag). B cells were irradiated with 1000 rads prior to use as APC.

[1,2] Statistically significant ($P<0.0125$).

* As for Table 5.1

Table 5.6 : Effect of anti-WNV antibodies on hyper-immune B cell-induced suppression of T cell proliferation[a].

S:R ratios [b]	B^{++++} + Ag	Concentration of added antibodies--mg/ml
2	*3,880 ± 159 [1]	0
1	13,567 ± 1481	
0.5	14,887 ± 2125	
0.25	24,928 ± 1617[2]	
0.125	15,952 ± 2417	

	Effect of added anti-WNV IgG	Effect of added anti-influenza IgG	
2	14,343 ± 1633[3]	30,181 ± 1432[5]	1
2	32,508 ± 2306	ND	2
2	38,776 ± 3559[4]	ND	4
2	6,525 ± 1446	5,569 ± 752	6
2	8,840 ± 1130	ND	8

[a] Data from the upper panel shows a typical effect of high APC (B^{++++}) numbers on the proliferative responses of $2x10^5$ WNV-specific $CD4^+$ T cells in the presence of a 1/8 dilution of WNV antigen stock (B^{++++} + Ag). Data from the lower panel shows the effect of added IgG antibodies against WNV and influenza on the T cell proliferation induced by B^{++++} cells at a S:R ratio of 2. Antibodies were added at the initiation of cultures in 20 µl aliquots. B^{++++} cells were obtained from mice receiving weekly injections of WNV over a period of four weeks.

[b] As for Table 5.1.

[1,3] Statistically significant ($P<0.0025$),

[2,4] Statitically significant ($P<0.01$),

[4,5] Statistically insignificant ($P>0.1$)

ND Not done.

* As for Table 5.1.

Table 5.7 : Reversal of B-cell-induced high-ligand suppression of T cell proliferation by anti-class II antibodies[1].

Antibody dilutions	Responses in the presence of anti-Ia^b antibodies		Responses in the presence of anti-Ia^k antibodies	
	B^{++++} + Ag	B^{++++} + Mo.Ag	B^{++++} + Ag	B^{++++} + Mo.Ag
No antibodies	* 5371 ± 156 [a]	978 ± 149		
1/200	18,537 ± 1721 [b]	1027 ± 85	11,388 ± 2757 [d]	1188 ± 145
1/1000	10,891 ± 1249 [c]	1682 ± 99	7400 ± 1027 [e]	1064 ± 93
1/5000	7430 ± 159	1434 ± 66	7652 ± 113	1032 ± 77
1/15,000	10,638 ± 2059	1232 ± 77	9508 ± 415	1062 ± 48

[1] Irradiated-hyper-immune B cells from C57BL/6 mice were co-cultured with WNV-immune $CD4^+$ T cells at a S:R ratio of 2, in the presence of varying dilutions of anti-Ia^b (IgG2b) or anti-Ia^k (IgG2b) monoclonal antibodies made from hybridoma cell supernatant stock and WNV antigen stock (B^{++++} + Ag) or mock control (B^{++++}+ Mo.Ag) at a 1/4 dilution.

[b,d & c,e] Statistically significant ($P<0.05$);

[a,b] Statistically significant ($P<0.0025$); [a,d] Statistically significant ($P<0.05$).

* As for Table 5.1.

The data are representative of one of the two experiments performed.

Table 5.8 : Reversal of LM-macrophage-induced suppression of T cell proliferation by anti-class II antibodies.[1]

S:R ratios [2]	M + Ag	Dilution of anti-Ia^b/D^b antibodies.
2	*13,504 ± 161 [a]	No antibodies
1	11,958 ± 944	
0.5	14,906 ± 1371	
0.25	34,807 ± 692 [b]	

	Anti-Ia^b antibodies M + Ag	Anti-D^b antibodies M + Ag	
2	3,634 ± 161 [k]	ND	1/30
2	12,611 ± 749 [c]	7,830 ± 355[f]	1/150
2	15,933 ± 818 [d]	10,459 ± 1178[g]	1/300
2	27,017 ± 814[e]	11,851 ± 1211[h]	1/1500

[1] Data from the upper panel shows a typical effect of high APC (macrophage) numbers on WNV-specific proliferation of immune $CD4^+$ T cells in the presence of a 1/4 dilution of WNV antigen stock (M + Ag). Data from the lower panel shows the effect of varying dilutions of specific (anti-Ia^b, IgG2b) & non-specific (control, anti-D^b, IgM) MAbs on the T cell proliferation induced by LM-macrophages at a S:R ratio of 2. Responses to mock antigens ranged from 379 ± 92 to 1483 ± 108 (cpm ± S.E.M.)

[2] As for Table 5.1.

b,e & c,f Statistically significant ($P < 0.0025$)

d,g Statistically significant ($P < 0.01$)

a,e, a,k & e,h Statistically significant ($P < 0.0005$)

* As for Table 5.1.; ND Not done.

The data are representative of one of the two experiments performed.

Table 5.9 : Reversal of LM-macrophage-induced suppression of T cell proliferation by Fab fragments of anti-class II antibodies [1].

S:R ratios [2]	M + Ag	Concentration of Fab/Fc proteins µg/ml
1	*5,206 ± 188 [a]	0
0.5	10,596 ± 348 [b]	
0.25	20,432 ± 1333	
0.125	36,089 ± 2362	

	Fab I^b M + Ag	Fab I^k M + Ag	Fc M + Ag	
1	6,268 ± 158	6,868 ± 561	4,977 ± 317	0.5
1	12,384 ± 179 [c]	5,091 ± 203 [d]	4,633 ± 251 [e]	1.00

[1] Data from the upper panel shows a typical effect of high APC numbers (macrophages) on WNV-specific T cell proliferation in the presence of a 1/4 dilution of WNV antigen stock (M + Ag). Data from the lower panel shows the effect of added Ia^b-& Ia^k-specific Fab & Fc proteins on the T cell proliferation induced by LM-macrophages at a S:R ratio of 1.

[2] As for Table 5.1.

[a,c] Statistically significant ($P<0.05$),

[b,c] Statistically insignificant ($P>0.2$),

[c,d] Statistically significant ($P<0.0025$),

[c,e] Statistically significant ($P<0.005$).

* As for Table 5.1.

The data are representative of one of the two experiments performed.

CHAPTER 6

GENERAL DISCUSSION

The immune system has evolved to provide an organism with mechanisms to combat pathogens and to maintain self-tolerance. Since the seminal observations that T cells recognize both native and denatured antigens (Gell and Benacerraf, 1959) and that APC are necessary for T cells to recognize antigens (Mosier, 1967) the phenomenon of antigen presentation has gradually become better understood. In the last two decades, considerable progress has been made in elucidating the mechanism(s) by which T cells recognize antigens. Work during the early 1970's established the importance of T cells in recovery from primary infections by viruses and other intracellular organisms (Blanden, 1974). The discovery of MHC-restriction further clarified the mechanisms by which T cells recognize foreign antigens (Zinkernagel and Doherty, 1974; Blanden *et al.*, 1975). The work of Ziegler and Unanue (1981) revealed that T cells recognize a processed form of antigen rather than the antigen per se and that APC fulfil the requirement of antigen processing and presentation in association with MHC molecules.

The concept of MHC-linked Ir genes further helped to explain the genetic basis of responsiveness (or non-responsiveness, Katz *et al.*, 1975; Benacerraf, 1978). Polymorphic residues from the peptide binding site of MHC molecules influence both the selection of appropriate peptides from processed antigens and MHC-dependent tolerance to self antigens during the generation of the T cell repertoire (Babbitt *et al.*, 1985; Kappler *et al.*, 1987 a,b). Two distinct antigen processing and

presentation pathways have been defined. Exogenously derived antigens are generally presented to class II-restricted T cells, whereas endogenously synthesized antigens are presented by class I-restricted T cells (Morrison *et al.*, 1986; Germain, 1986). Although the molecular mechanisms in the latter pathway are not fully understood, the finding that viral antigens need not be expressed on infected cell surfaces for Tc cell recognition gave fundamental insight into the nature of antigens recognized by Tc and Th cells (Townsend, 1988 a,b).

The crystallographic investigation of the structure of HLA-A2 molecules, showing a membrane-distal elongated cleft, the sides formed by two α helices and the bottom formed by a β pleated sheet as a probable binding site for processed antigens, constituted a structural confirmation of previous functional data (Bjorkman *et al.*, 1987 b).

An understanding of T cell responses to viral antigens should provide insights into potential problems with purified protein vaccines in human populations. This thesis concerns the identification of flavivirus antigens by MHC class II-restricted $CD4^+$ T cells. The relative efficacy of different APC populations such as peritoneal exudate macrophages, B cells and SDC in presenting WNV antigens was compared. The influence of Ir genes and background genes on WNV-and Kunjin-specific T cell responses was determined. The nature of Kunjin proteins which are a source of epitopes for $CD4^+$ T cells were partly defined by a panel of recombinant vaccinia viruses encoding various Kunjin polypeptides. Finally, the effects of high APC number-induced suppression of T cell proliferation observed during the course of these investigations were evaluated. In this Chapter the findings

of key experiments reported in this thesis together with the general ideas and concepts that have emerged from the work are discussed.

As a result of MHC-restricted antigen presentation only a particular set of antigen-specific T lymphocyte clones are activated and expanded by proliferation. However, *in vitro* analysis of antigen-induced T cell activation is affected by several key variables. These include the frequency and heterogeneity of responding T cell clones, the responses of bystander populations to the products of activated cells, the type and number of APC used and the nature and concentration of antigen used. Although the use of antigen-specific, cloned T cell populations is useful for investigations of fine antigen-specificity, the differentiated properties of such clones may not permit extrapolation to the responses of polyclonal populations and many clones would be needed to fully elucidate the spectrum of epitopes involved in the polyclonal responses of the animal. To avoid these problems, the variables pertinent to *in vitro* WNV-specific $CD4^+$ T cell responses against various WNV antigen were established in Chapter 2. Of the various kinds of antigen preparations tested for pulsing APC, lysates prepared from WNV-infected Vero cells induced the best responses from immune $CD4^+$ T cells. A modified T cell proliferation assay using NMS was developed to reduce the levels of background proliferation occurring mainly due to media containing FCS. This assay involved initial APC and T cell culturing in the presence of WNV antigen and NMS for 3 days, followed by a further 2 days culture in the media containing EL-4 supernatant as an exogenous source of IL-2 and a variety of other T cell growth-promoting factors.

Modification of the T cell proliferation assay led to a substantial reduction in background proliferation.

Antigen-presenting cells provide a ligand for T cells in the form of a processed antigenic peptide complexed with a MHC molecule. The role of macrophages, B cells and DC as APC both *in vivo* and *in vitro* has now been well-established. These cells differ in their ability to take up, process and present soluble and particulate antigens. These APC also show qualitative and quantitative differences in the levels of expressed MHC class II antigens (Cullen, *et al.*, 1981; Nussenzweig *et al.*, 1981). Therefore, it was predicted that these APC would differ in their ability to present flavivirus antigens.

Macrophages express class II MHC antigens facultatively rather than constitutively. After expressing cell-surface Ia antigens, murine peritoneal macrophages became excellent APC as described in Chapter 3. The improvement in the efficiency of antigen presentation by macrophages in the presence of specific antibody *in vitro* indicates that in secondary responses *in vivo*, pre-existing antibodies may amplify immune reponses by exerting augmenting effects on $CD4^+$ T cells and thus, indirectly, regulate their own synthesis (Cohen *et al.*, 1973; Celis *et al.*, 1984).

The requirement of lower WNV antigen concentrations for populations of B^{++++} cells than for B_0 or B_{14} cells to function optimally as APC and the inhibition of their antigen-presenting function by WNV-specific antibodies implied that antigen-specific B cells were the predominant APC in the B^{++++} cell population. On a cell population basis, LM macrophages were the most efficient APC when compared to B cells derived from naive or 14 day-

WNV-primed mice and SDC. Therefore, LM macrophages were invariably used as APC for evaluating the responses of WNV or Kunjin-primed mice from MHC-disparate strains. Since macrophages and B cells differ in their APC function as described above, in certain experiments reported in Chapter 4, B_{14} cells were also used as APC.

Understanding the genetic basis of responsiveness should give insight into the potential problems involved in development of vaccines expressing several peptides, thus enabling rational development of multi-epitope vaccines, which should be capable of eliciting T cell responses in most individuals in outbred populations. Data presented on this question in Chapter 4 revealed two interesting features.

Firstly, the lysate preparations derived from CV1 cells infected with Kunjin-VV recombinants, although containing several potential antigenic determinants, predominantly stimulated T cells recognizing epitopes derived from structural and membrane-associated non-structural protein and, to some extent, cytosolic non-structural protein (NS5). As discussed in Chapter 4, apparent bias of class II-restricted T cells for the recognition of structural and membrane-associated, non-structural proteins can be explained on the basis of relative availability of different viral proteins for T cell recognition. Membrane-associated proteins follow a pathway through ER and Golgi, and the vesicles containing these proteins finally fuse with the plasma membrane and therefore it is unlikely that they are subjected to intracellular proteolysis. In contrast, the chance of cytosolic non-structural proteins being degraded by cellular enzymes are likely to be greater. This bias may be amplified as

the immune response develops. It may be that during the early phase of the immune response, MHC class II+ macrophages take up and present antigens to CD4+ T cells or release antigenic fragments, which then bind to DC. At this stage, these APC may present all potential epitopes to the resting T cells. However, in a later phase of the immune response, when antigen concentration is low, antibody-dependent uptake of antigen may be dominant. Thus, if antibody responses are biased towards membrane-associated viral proteins, these proteins may be the dominant source of epitopes for CD4+ T cells. This reasoning is based on the observation that specific sIg present on B cell APC is involved in the efficient uptake of antigen even at 10,000-fold lower concentration than non-sIg-mediated pathways (Lanzavecchia, 1985) and the augmented uptake of antigen by macrophages in the presence of antibody discussed above.

The observed bias towards the recognition of structural and membrane-associated non-structural proteins by CD4+ T cells is consistent with the observations that the majority of anti-flavivirus antibodies recognize surface structural glycoproteins and NS1 (Schlesinger *et al.*, 1985, 1986; Zhang *et al.*, 1988). It may be speculated that in anti-μ suppressed mice there could be a greater proportion of the CD4+ T cell response directed towards the non-structural proteins. The finding that with macrophage APC, Kunjin-immune CBA T cells gave higher responses to 1022 than 1031 (Fig. 4.2) but with B cell APC, the responses to 1031 were stronger than 1022 (Table 4.2) suggest that, 1) preferential uptake of membrane associated viral antigens by a proportion of antigen-specific B cells from the APC population presumably resulted in the selective activation of CD4+ T cells specific for

these antigens, and, 2) macrophages and B cells display a different spectra of peptides for T cell activation.

The relative contribution of different virus-specified structural proteins in MHC class II-restricted T cell responses awaits further analysis by constructing a more extensive panel of recombinant vectors expressing individual proteins and smaller peptides, and ultimately the use of appropriate synthetic peptides.

Secondly, the finding that regardless of haplotype, the responses to 1024, (which encompasses the non-structural genes of Kunjin virus including those encoded by 1022, Chapter 4), were lower than those against 1022 means that the mere presence of large numbers of potential antigenic determinants is not enough to guarantee an immune response of $CD4^+$ T cells. Differences in the processing of 1022 and 1024, as discussed in detail in Chapter 4, possibly account for these findings.

The responses of WNV-and Kunjin-immune T cells varied amongst mice with different MHC haplotypes. Two types of mutually non-exclusive models have been suggested for the influence of MHC Ir genes on T cell responses. The models which propose that Ir genes function at the level of MHC-peptide interaction ('antigen-presentation' models) assume that non-responsiveness is due to failure of a particular MHC molecule to bind to a particular antigen-derived peptide (Babbitt *et al.*, 1985). In such models no constraints are placed on T cell repertoire. Alternatively, repertoire selection models hold that MHC Ir genes shape the T cell repertoire by selection processes within and sometimes outside the thymus. Of particular interest is the influence of a particular allele at a given locus on the

responses to an antigen in association with an allelic product of another MHC-encoding locus (immunodominance or cross-tolerance). Mullbacher *et al* (1983) have shown that the phenomenon in which the presence of the K^k allele inhibited Tc cell responses to vaccinia virus in association with D^b, was due to K^k cross-reacting with vaccinia plus D^b. Thus self tolerance to K^k suppressed Tc cell clones potentially reactive to vaccinia-D^b (discussed in Chapter 1). The mechanisms underlying the Ir gene effects on MHC class II-restricted responses to WNV and Kunjin antigens observed here are not yet clear and will require substantial further experimentation.

In analyzing the $CD4^+$ T cell proliferative responses from different inbred mouse strains, the influence of background genes was manifested in low virus-specific responses from strains of C57BL background (Fig. 4.2). This phenomenon was apparently caused by suppression of T cell responses by APC numbers optimal for maximum reactivity in other mouse strains via high ligand suppression. These observations suggest the possibility that background genes affect the processing and presentation of peptides. In addition, qualitative and quantitative differences in cell surface adhesion molecules present on both APC and T cells that are involved in the formation of stable conjugates may also be involved.

In optimizing the numbers of APC in Chapters 2, 3 and 4 it was observed that high APC numbers suppressed proliferative $CD4^+$ T cell responses. In Chapter 5, this phenomenon of high APC-induced suppression was investigated. Anti-Ia antibodies (or their Fab fragments) significantly reduced the suppression, presumably by blocking access of TcR to class II MHC.

Furthermore, using suppressive numbers of APC, there was a progressive increase in T cell proliferation as WNV antigen concentration was decreased. These findings suggested that high concentrations of both available MHC and the nominal antigen contribute towards the induction of suppression i.e., it was caused by excessive concentrations of MHC-antigen complexes, the ligands for TcR, as previously demonstrated with T cell clones by Matis *et al* (1983). In order to consider the possible relevance of high ligand-induced suppression to immunological tolerance, it is appropriate to discuss the quantitative aspects of T cell activation.

The signal received by a single T cell in contact with APC depends upon, (1) affinity of TcR for ligand, (2) the concentrations of TcR and CD4 (or CD8) present on the T cell, and, (3) the concentration of available ligand (MHC + antigenic peptide complex) on the APC. Since (1) and (2) are constant for a single T cell at a given time point, the variable that determines the signal for T cell activation is the concentration of ligand as shown in this thesis for $CD4^+$ T cell populations and for clones by Matis *et al* (1983). Activation will occur only when a certain threshold of signal strength is exceeded (self evident) but whether the response is an all or none phenomenon, (Blanden *et al.*, 1987) or graded according to signal strength is unknown. However, the data presented here and by others (Matis *et al.*, 1983; Lamb *et al.*, 1983) indicate that if ligand concentration is too high, the response is reduced in clones and populations, which may mean that it is terminated in certain single cells. Assuming an all or none response for both activation and termination for the purpose of discussion, then if response (e.g proliferation) is

plotted against the concentration of ligand on an arbitrary scale, then the response of a single cell can be represented by a histogram (A) (Fig.6.1). Extrapolating this to a given clone of T cells, where the affinity of different cells to a given ligand is constant by definition, but the concentration of TcR and CD4 (or CD8) presumably vary, the quantity of ligand required for achieving the signal thresholds for activation and termination of individual cells will vary. Therefore, some cells will be activated at low concentrations of ligand, others at high; this is represented by histograms B, C, D, E, F and G (Fig. 6.1). Assuming a normal distribution for the varying TcR and CD4 concentrations, the response of the clone should be a typical bell-shaped curve, represented as J in Fig. 6.1, because more individual cells will be responding a ligand concentration in the central part of the concentration range for the clone. In fact, such curves have been published by Matis *et al* (1983) and Lamb *et al* (1983), although whether the contributions of single cells are histograms or some other form is unknown. In a population of T cells, which consists of different clones with varying affinity their ligands, affinity of TcR will be an additional variable, together with TcR and CD4 (or CD8) concentrations, contributing to differences between cells in the population. Whether affinity has a bigger influence than receptor concentrations or ligand concentration required for activation and termination is unknown. However, data presented in this thesis, strongly suggests that T cell populations also give a bell-shaped curve (K in Fig. 6.1) when response is plotted against ligand concentration. This pattern is consistent with the data from Tables 5.1, 5.5 and 4.6.

Does the phenomena of supra-optimal ligand-induced T cell unresponsiveness fulfil the criteria of immunological tolerance ? The evidence for clonal deletion in the thymus is well-documented. As discussed in Chapter 1, Kappler *et al* (1987, a,b) showed that mature Vβ17a$^+$ T cells (reacting preferentially with I-E molecules) were selectively absent in mice expressing I-E. The question as to whether a similar mechanism of deletion occurs in the periphery after encounter with high ligand (MHC associated with foreign peptide or hapten), is more contentious. However, the contentious point is not whether responses of T cells are depressed by high ligand concentration which is well-documented *in vivo* (Claman *et al.*, 1980; Monroe *et al.*, 1984) and *in vitro* (Matis et al., 1983; this thesis, Chapter 5) but whether the low responses are due to clonal deletion or anergy.

Recently, Rammensee *et al* (1989) showed that, when adult mice expressing minor lymphocyte antigen b (Mls^b), were tolerized for Mls^a antigens by a single i.v. injection of 10^7 splenocytes of *Mls^a* strain of mice (of the same MHC haplotype), T cells recognizing Mls^a antigens were present in unchanged numbers. These cells were "anergic" in that they were unable to proliferate, secrete IL-2 or respond to exogenous IL-2 when stimulated with Mls^a cells *in vitro*.

In the present studies, the suppression of T cell proliferation at high S:R ratios occurred even in the presence of co-stimulatory factors (EL-4 supernatant). This suggests that it is not a phenomenon like that described by Jenkins and Schwartz (1987) and attributed to a lack of APC accessory signals needed in addition to TcR occupancy by ligand. Therefore it would be of interest to investigate whether these cells could respond at later

times given optimal APC numbers, whether they were killed (deleted), or alive but "anergic".

Alternative mechanisms of the phenomenon described here include, 1) supra-optimal concentration of ligand may selectively stimulate TH1 clones of $CD4^+$ T cells to produce high levels of IFN-γ, which in turn inhibits the proliferation of TH2 cells (Gajewski and Fitch, 1988). However, as discussed in Chapter 3, this is a possible explanation for the data in this thesis only when the frequency of TH1 is much lower than TH2. Alternatively, high concentrations of ligand might increase the signal to TcR resulting in the activation of altered sets of lymphokine-secreting genes e.g. IFN-γ (Matis *et al.*, 1983), and, 2) high concentrations of antigen may prevent redistribution of TcR into the region of the T cell surface that is in contact with the APC, this being essential for signalling events in activation of T cells (Kupfer *et al.*, 1987). In an analogous situation, Becker *et al* (1973) showed that higher concentration of anti-IgE antibodies, which in fact redistribute the surface IgE most effectively, caused inhibition of histamine release from human basophils.

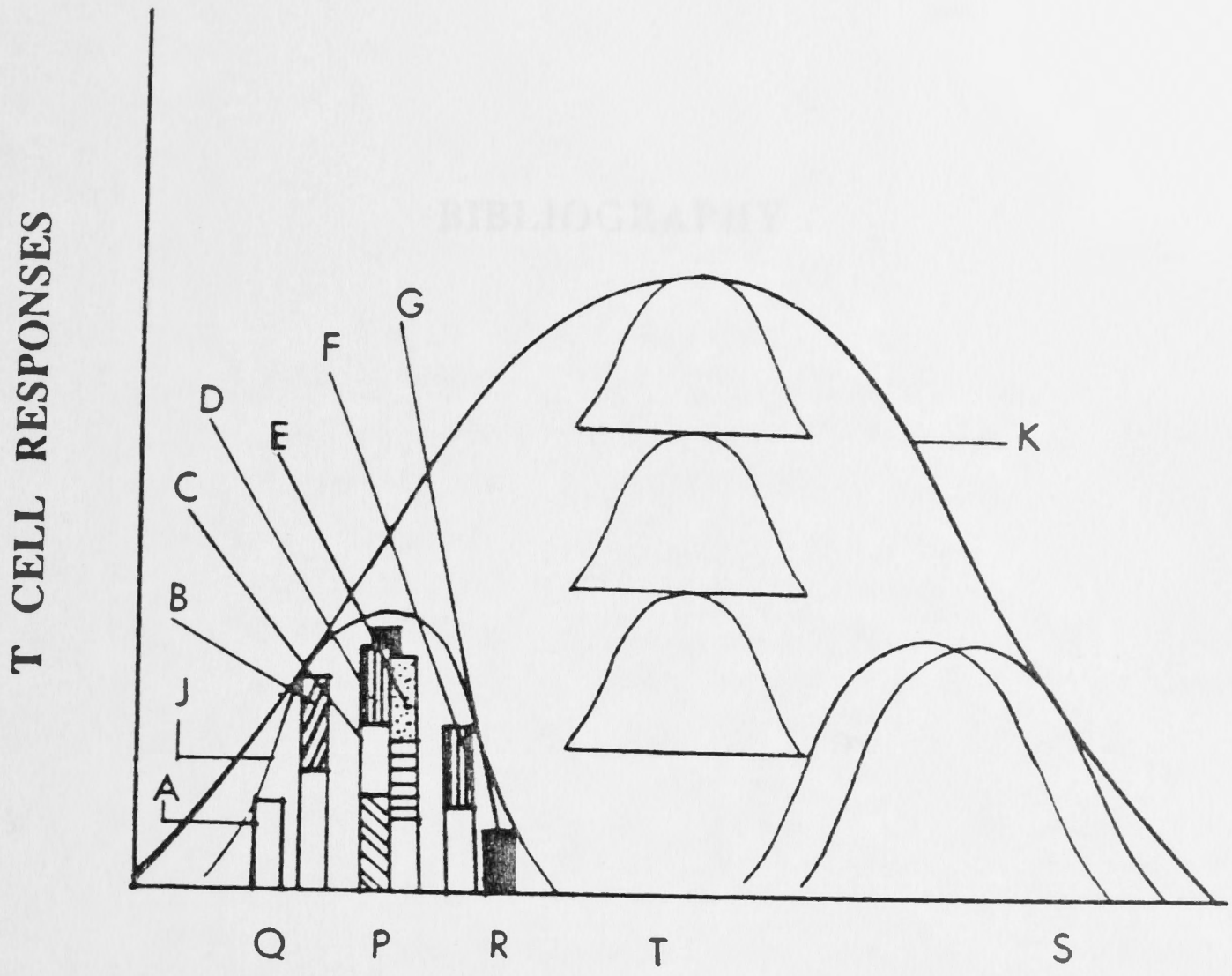
Unresponsiveness of $CD4^+$ T cells to WNV antigen at high APC numbers, presumably due to high ligand concentration, has possible implications in the pathogenesis of infections with viruses and other intracellular parasites. For a parasite, the expression of supra-optimal concentration of ligand would provide a means of evading T cell recognition. Interestingly, evidence for the upregulation of MHC class I and class II antigens on cultured cells by WNV has been published by Liu *et al* (1988) for astrocytes and King *et al.*, (1989) for fibroblasts. If the ligand concentration on WNV virus-infected cells were above the

threshold concentration required for high ligand suppression of responses, then reduced T cell response would result. Whether such events cause real effects *in vivo* remains to be determined.

Fig. 6.1

Curve J represents the response of a given T cell clone in which responses of individual cells, depending upon ligand concentration (Φ-S) required for activation and termination will vary, i.e., some cells will be activated at low concentration of ligand, others at high. This is represented by a series of histograms (A to G, assuming that response is 'all or none' phenomenon); however, the majority of cells will respond to a ligand in the central part (P) of the ligand concentration range for the clone. At a population level, clones with varying affinities and varying TcR and CD4 (or CD8) concentrations, will demand different concentrations of ligand for activation and termination. Therefore, the distribution pattern of these clones will be similar to individual cells within a clone, i.e., majority of clones will be activated and terminated in the central part (T) of the ligand concentration range for the population. This pattern of the population is represented by curve K.

Fig. 6-1



BIBLIOGRAPHY

Acuto, O., Hussey, R.E., Fitzgerald, K.A., Protentis, J.P., Meuer, S.C., Schlossman, S.F. and Reinhberz, E.L. 1983. The human T cell receptor: appearance in ontogeny and biochemical relationship of α and β subunits on IL-2 dependent clones and T cell tumors. *Cell* 37: 717.

Adorini, L., Muller, S., Cardinaux, f., Lehmann, P.V., Falcioni, F. and Nagy, Z.A. 1988. *In vivo* competition between self peptides and foreign antigens in T-cell activation. *Nature* 334: 623.

Allan, J.E. and Doherty, P.C. 1986. Stimulation of helper/delayed type hypersensitivity T cells by flavivirus infection: Determination by macrophage procoagulant assay. *J. Gen. Virol* . 67: 39.

Allen, H., Wraith, D., Pala, P., Askonas, B., and Flavell, R.A. 1984. Domain interactions of H-2 class I antigens alter cytotoxic T-cell recognition sites. *Nature* 309: 279.

Allen, P. 1987. Antigen processing at the molecular level. *Immunol. Today*. 8: 270.

Allison, J., McIntyre, B., and Bloch, D. 1982. Tumor specific antigen of murine T-lymphoma defined with monoclonal antibody. *J. Immunol.* 129: 2293.

Andersson, B., Skoglund, A.C., Ronnholm, M., Lindsten, T., Lamon, E.W., Collisson, E.W. and Walia, A.S. 1981. Functional aspects of IgM and IgG Fc receptors on murine T lymphocytes. *Immunol Rev*. 56: 5.

Apple, R.J., Domen, P.L., Muckerheide, A. and Michael, J.G. 1988. Cationization of protein antigens IV Increased antigen uptake by antigen presenting cells. *J. Immunol.* 140: 3290.

Asanso, Y. and Hodes, R.J. 1983. T cell regulation of B cell activation. Cloned Lyt $1^{+}2^{-}$ T suppressor cells inhibit the major histocompatibility complex-restricted interaction of helper T cell with B cells and/or accessory cells. *J. Exp. Med.* 158: 1178.

Ashwell, J.D., DeFranco, A.L., Paul, W.E. and Schwartz, R.H. 1984. Antigen presentation by resting B cells: radiosensitivity of the antigen presentation function and two distinct pathways of T cell activation. *J. Exp. Med.* 159: 881.

Austyn, J.M. 1987. Lymphoid dendritic cells. *Immunology 62*: 161.

Babbitt, B.P., Allen, P.M., Matsueda, G., Haber, E. and Unanue, E.R. 1985. The binding of immunogenic peptides to Ia histocompatibility molecules. *Nature.* 317: 359.

Babbitt, B.P., Matsueda, G., Haber, E., Unanue, E.R. and Allen, P.M. 1986. Antigenic competition at the level of peptide-Ia binding. *Proc. Natl. Acad. Sci. USA.* 83: 4509.

Barcinski, M.A., and Rosenthal, A.S. 1978. Immune response gene control of determinant selection I. Intramolecular mapping of the immunogenic sites on insulin recognized by guinea pig T and B cells. *J. Exp. Med.* 145: 726.

Basten, A., Warner, N.L. and Mandel, T. 1972. A receptor for antibody on B cells. II. Immunochemical and electron microscopy characteristics. *J. Exp. Med.* 135: 627.

Becker, K.E., Ishizaka, T., Metzger, H. and Grimley, P.M. 1973. Surface IgE on human basophils during histamine release. *J. Exp. Med.* 138: 394.

Beller, D.I. 1984. Functional significance of the regulation of macrophage Ia expression. *Eur. J. Immunol.* 14: 138.

Beller, D.I. and Unanue, E.R. 1981. Regulation of macrophage populations. II. Synthesis and expression of Ia antigens by peritoneal macrophages is a transient event. *J. Immunol.* 126: 263.

Beller, D.I., Kiely, J-M. and Unanue, E.R. 1980. Regulation of macrophage populations. I. Preferential induction of Ia-rich peritoneal exudates by immunologic stimuli. *J. Immunol.* 124: 1426.

Benacerraf, B. 1978. A hypothesis to relate the specificity of lymphocytes and the ability of I-region-specific Ir genes in macrophage and B lymphocytes. *J. Immunol.* 120: 1809.

Benoist, C.O., Mathis, D.J., Kanter, M.R., Williams, V.E. II and McDevitt, H.O. 1983. The murine Ia α chains, E_α and A_α, show a surprising degree of sequence homology. *Proc. Natl. Acad. Sci.* 80: 534.

Berg, L.J., Pullen, A.M., Groth, B.F., Mathis, D., Benoist, C. and Davis, M.M. 1989. Antigen/MHC-specific T cells are predominantly exported from the thymus in the presence of their MHC ligand. *Cell* 58: 1035.

Bevan, M.J. and Fink, P.J. 1978. The influence of thymus H-2 antigens on the specificity of maturing killer and helper cells. *Immunol. Rev.* 42: 3.

Bhan, A.K., Nadler, L.M., Stashenko, P., McCluskey, R.T., and Schlossman, S.F. 1981. Stages of B cell differentiation in human lymphoid tissue. *J. Exp. Med.* 154: 737.

Bierer, B., Peterson, A., Gorga, J.C., Herrmann, S.H., Burakoff, S.J. 1988. Synergistic T cell activation via the physiological ligands for CD2 and the T cell receptor. *J. Exp. Med.* 168: 1145.

Bierer, B.E., Sleckman, B.P., Ratnofsky, S.E. and Burakoff, S.J. 1989. The biologic roles of CD2, CD4 and CD8 in T cell activation. *Ann. Rev. Immunol.* 7: 579.

Bjorkman, P.J., Saper, M.A., Samaroui, B., Bennett, W.S., Strominger, J.L. and Wiley, D.C. 1987 a. Structure of the human class I histocompatibility antigen, HLA-A2. *Nature* 329: 506.

Bjorkman, P.J., Saper, M.A., Samaroui, B., Bennett, W.S., Strominger, J.L. and Wiley, D.C. 1987 b. The foreign antigen binding site and T cell recognition regions of class I histocompatibility antigens. *Nature* 329: 512.

Blanden, R.V. 1974. T cell response to viral and bacterial infection. *Transp. Rev.* 19: 56.

Blanden, R.V. and Ashman, R.B. 1985. Selection of pre-T cells in the thymus by unique combinations of major and minor histocompatibility antigens. *Mol. Immunol.* 22: 827.

Blanden, R.V., Hodgkin, P.D., Hill, A., Sinikas, V.G. and Mullbacher, A. 1987. Quantitative considerations of T cell activation and self-tolerance. *Immunol. Rev.* 98: 75.

Blanden, R.V., Doherty, P.C., Dunlop, M.B.C., Gardner, I.E., Zinkernagel, R.M. and David, C.S. 1975. Genes required for cytotoxicity against virus-infected target cells in K and D regions of the H-2 complex. *Nature*. 254: 269.

Bluestone, J.A. and Matis, L.A. 1989. TcR γδ cells - Minor redundant T cell subset or specialized immune system component ? *J. Immunol.* 142: 1785.

Bluestone, J.A., Cron, R.Q., Cotterman, M., Houlden, B.A. and Matis, L. 1988. Structure and specificity of T cell receptor γ/δ on major histocompatibility complex antigen-specific $CD3^+$, $CD4^-CD8^-$ T lymphocytes. *J. Exp. Med.* 168: 1899.

Born, W., Miles, C., White, J., O' Brien, R., Freed, J.H., Marrack, P., Kappler, J. and Kubo, R.T. 1987. Peptide sequence of T cell receptor δ and γ chains are identical to predicted X and γ proteins. *Nature* 330: 572.

Braciale, T.J., Braciale, V.L., Winkler, M., Stroynowski, I., Hood, L., Sambrook, J. and Gething, M.J. 1987. On the role of the transmembrane anchor sequence of influenza haemagglutinin in target-cell recognition by class I MHC-restricted, haemagglutinin-specific cytolytic T lymphocytes. *J. Exp. Med.* 166: 678.

Brandt, W.E. 1988. Current approaches to the development of dengue vaccines and related aspects of the molecular biology of flaviviruses. *J. Infect. Dis.* 157: 1105.

Brett, S.J., Cease, K.B. and Berzofsky, J.A. 1988. Influences of antigen processing on the expression of the T cell repertoire: evidence for MHC-specific hindering structures on the products of processing. *J. Exp. Med.* 168: 357.

Brinton, M.A. 1986. Replication of flaviviruses. In *"The Togaviridae and flaviviridae" Ed. Schlesinger, S. and Schlesinger, M.J. Plenum Press, New York and London,* pp. 327.

Brown, J.H., Jardetzky, T., Saper, M.A., Samraoui, B., Bjorkman, P.J. and Wiley, D.C. 1988. A hypothetical model of the foreign antigen binding site of Class II histocompatibility molecules. *Nature* 332: 845.

Brown, M.F., Cook, R.G., Van, M. and Rich, R.R. 1984. Cloned human T cells synthesize Ia molecules and can function as antigen-presenting cells. *Human Immunol.* 11: 219.

Burke, D.S. and Morrill, J.C. 1987. Levels of interferon in the plasma and cerebrospinal fluid of patients with acute Japanese encephalitis. *J. Infect. Dis.* 155: 797.

Buus, S., Sette, A., Colon, S.N. and Grey, H.M. 1988. Autologous peptides constitutively occupay the antigen binding site on Ia. *Science* 242: 1045.

Buus, S. and Werdelin, O. 1986. A group-specific inhibitor of lysosomal proteinases selectively inhibits both proteolytic degradation and presentation of the antigen DNP- poly-L-lysin by guinea pig accessory cells to T cells. *J. Immunol.* 136: 452.

Buus, S., Colon, S., Smith, C., Freed, J.H., Miles, C., and Grey, H.M. 1986. Interaction between a "processed" ovalbumin peptide and Ia molecules. *Proc. Natl. Acad. Sci. USA.* 83: 3968.

Buus, S., Sette, A., Colon, S.M., Miles, C., and Grey, H.M. 1987. The relation between major histocompatibility complex (MHC) restriction and the capacity of Ia to bind immunogenic peptides. *Science.* 235: 1353.

Calderon, J. and Unanue, E.R. 1974. The release of antigen molecules from macrophages: characterization of the phenomena *J. Immunol.* 112: 1804.

Castle, E., Leidner, U., Nowak, T., Wengler, G. and Wengler, G. 1986. Primary structure of the West Nile Flavivirus genome region coding for all nonstructural proteins. *Virology*. 149: 10.

Celis, E., Karr, R.W., Dietzschold, B., Wunner, W.H. and Koprowski, H. 1988 a. Genetic restriction and fine specificity of human T cell clones reactive with rabies virus. *J. Immunol.* 141: 2721.

Celis, E., Ou, D. and Otvos, L. 1988b. Recognition of hepatitis B surface antigen by human T lymphocytes. Proliferative and cytotoxic responses to a major antigenic determinant defined by synthetic peptides. *J. Immunol.* 140: 1808.

Celis, E. Zurawski, V.R.Jr. and Chang, T.W. 1984. Regulation of T cell function by antibodies: Enhancement of the responses of human T cell clones to hepatitis B surface antigen by antigen-specific monoclonal antibodies. *Proc. Natl. Acad. Sci. USA*. 81: 6846.

Ceredig, R., Jenkinson, E.J., MacDonald, H.R. and Owen, J.J.T. 1982. Development of cytotoxic T lymphocyte precursors in organ-cultured mouse embryonic thymus rudiments. *J. Exp. Med.* 155: 617.

Ceredig, R., Lowenthal, J.W., Nabholz, M. and MacDonald, H.R. 1985. Expression of interleukin-2 receptors as a differentiation marker on intrathymic stem cells. *Nature*. 314: 98.

Chain, B., McCafferty, I., Wallace, G. and Askenase, P.W. 1987. Improvement of the *in vitro*. T cell proliferation assay by a modified method that separates the antigen recognition and IL-2 dependent steps. *J Immunol Methods*. 99: 221.

Chesnut, R.W. and Grey, H.M. 1981. Studies on the capacity of B cells to serve as antigen-presenting cells. *J. Immunol.* 126: 1075.

Chesnut, R.W. and Grey, H.M. 1986. Antigen presentation by B cells and its significance in T-B interactions. *Adv. Immunol.* 39: 51.

Chesnut, R.W., Colon, S. and Grey, H.M. 1982. Requirements for the processing of antigen by antigen presenting B cells. I. Functional comparison of B tumors and macrophages. *J. Immunol.* 129: 2382.

Chesnut, R.W., Colon, S.M. and Grey, H.M. 1982. Antigen-presentation by normal B cells, B cell tumors and macrophages. Functional and biochemical comparison. *J. Immunol.* 128: 1764.

Chisen, Y.H., Gascoigne, N.R.J., Kavaler, J., Lee, N.E. and Davis, M.M. 1984. Somatic recombinations in a murine T-cell receptor gene. *Nature* 309: 322.

Claman, H.N., Miller, S.D., Conlon, P.J. and Moorhead, J.W. 1980. Control of experimental contact sensitivity. *Adv. Immunol.* 30: 121.

Cohen, B.E., Rosenthal, A.S. and Paul, W.E. 1973. Antigen macrophage interaction. II. Relative roles of cytophilic antibody and other membrane sites. *J. Immunol.* 111: 820.

Coia, G., Parker, M.D., Speight, G., Byrne, M.E. and Westaway, E.G. 1988. Nucleotide and complete amino acid sequences of Kunjin Virus: Definitive gene order and characteristics of the virus-specified proteins. *J. Gen. Virol.* 69: 1.

Cole, G.A., Nathanson, N. and Prendergast, R.A. 1972. Requirement for theta bearing cells in lymphocytic choriomeningitis virus induced central nervous system disease. *Nature* 238: 335

Coligan, J.E., Kindt, T.J., Vehara, M., Martinko, J. and Nathenson, S.G. 1981. Primary structure of murine transplantation antigen. *Nature* 291: 35.

Corradin, G. and Chiller, J.M. 1979. Lymphocyte specificity to protein antigens. II. Fine specificity of T-cell activation with cytochrome c and derived peptides as antigenic probes. *J. Exp. Med.* 149: 436.

Cowing, C. and Chapdelaine, J.M. 1983. T cells discriminate between Ia antigens expressed on allogenic accessory cells and B cells: A potential function for carbohydrate side chains on Ia molecules. *Proc. Natl. Acad. Sci. USA.* 80: 6000.

Cresswell, P. 1985. Intracellular class II HLA-antigens are accessible to transferrin-neuraminidase conjugates internalized by receptor-mediated endocytosis. *Proc. Natl. Acad. Sci. USA.* 82: 8188.

Cullen, S.E., Kindle, C.S., Shreffler, C.D. and Cowing, C. 1981. Differential glycosylation of murine B cell and spleen adherent cell Ia antigens. *J. Immunol.* 127: 1478.

Dalgarno, L., Trent, D.W. Strauss, J.H. and Rice, C.M. 1986. Partial nucleotide sequence of the Murray Valley encephalitis virus genome. Comparison of the encoded polypeptides with yellow fever virus structural and non-structural proteins. *J. Mol. Biol.* 187: 309.

Davis, M.M., Chisen, Y.H., Gascoigne, R.J. and Hedrick, S.M. 1985. A murine T cell receptor gene complex: isolation, structure and rearrangement. *Immunol. Rev.* 81: 235.

DeLisi, C. and Berzofsky, J.A. 1985. T-cell antigenic sites tend to be amphipathic structures. *Proc. Natl. Acad. Sci. USA.* 82: 7048.

Dembic, Z., Haas, W., Zamoyska, R., Parnes, J., Steinmetz, M. and von Boehmer, H. 1987. Transfection of the CD8 gene enhances T-cell recognition. *Nature* 326: 510.

Doyle, C. and Strominger, J.L. 1987. Interaction between CD4 and class II MHC molecules mediates cell adhesion. *Nature* 330: 256.

Drexhage, H.A., Mullink, H., de Groot, J., Clarke, J. and Balfour, B.M. 1979. A study of cells present in peripheral lymph of pigs with special reference to a type of cell resembling the Langerhans cells. *Cell Tissue Res.* 202: 407.

Ey, P.L., Prowse, S.J. and Jenkin, C.R. 1978. Isolation of pure IgG1, IgG2a and IgG2b immunoglobulins from mouse serum using protein A-sepharose. *Immunochemistry.* 15: 429.

Fagraeus, A., Ehrnst, A., Klein, E., Patarroyo, M., Goldstein, G. 1982. Characterization of blood mononuclear cells reacting with K562 cells after yellow fever vaccination. *Cell Immunol.* 67: 37

Falo, L.D., Jr., Benacerraf, B., Rothstein, L., and Rock, K.L. 1987. Cerulenin is a potent inhibitor of antigen processing by antigen-presenting cells. *J. Immunol.* 139: 3918.

Farrer, J.J., Farrar, J.F., Simon, P.L., Hilfiker M.L., Stadler B.M. and Farrar, W.I. 1980. Thymoma production of T cell growth factor (interleukin-2). *J. Immunol.*. 125: 2555.

Fowlkes, B.J., Schwartz, R.H. and Pardoll, D.M. 1988. Deletion of self-reactive thymocytes occurs at a $CD4^{+}8^{+}$ precursor stage. *Nature.* 334: 620.

Frohman, M. and Cowing, C. 1985. Presentation of antigen by B cells: Functional dependence on radiation dose interleukins, cellular activation and differential glycosylation. *J. Immunol.* 134: 2269.

Gajdosova, E., Mayer, V. and Oravec, C. 1980. Cross-reactive killer T lymphocytes in a flavivirus infection. *Acta Virol.* 24: 291.

Gajdosova, E., Oravec, C. and Mayer, V. 1981. Cell mediated immunity in flavivirus infections I. Induction of cytotoxic T lymphocytes in mice by an attenuated virus from the Tick-borne encephalitis complex and its group-reactive character. *Acta. Virol.* 25: 10.

Gay, D., Maddon, P., Sekaly, R., Talle, M.A., Godfrey, M., Long, E., Goldstein, G., Chess, L., Axel, R., Kappler, J., Marrack, P. 1987. Functional interaction between human T-cell protein CD4 and the major histocompatibility complex HLA-DR antigen. *Nature* 328: 626.

Gell, P.G. and Benacerraf, B. 1959. Studies on hypersensitivity. II. Delayed hypersensitivity to denatured proteins in guinea pigs. *Immunology.* 2: 64.

Germain, R. and Malissen, B. 1986. Analysis of the expression and function of class II major histocompatibility complex-encoded molecules by DNA mediated gene transfer. *Ann. Rev. Immunol.* 4: 281.

Germain, R.N. 1986. The ins and outs of antigen processing and presentation. *Nature* 322: 687.

Gollins, S.W. and Porterfield, J.S 1986. A new mechanism for neutralization of enveloped virus by antiviral antibody. *Nature* 321: 244.

Gollins, S.W. and Porterfield, J.S. 1984. Flavivirus infection enhancement in macrophages: Radioactive and biological studies on the effect of antibody on viral fate. *J. Gen. Virol.* 65: 1261.

Gould, E.A., Buckley, A., Groeger, B.K., Cane, P.A. and Doenhoff, M. 1987. Immune enhancement of yellow fever virus neurovirulence for mice: studies of mechanisms involved. *J. Gen. Virol.* 68: 3105.

Guagliardi, L.E., Koppelman, B., Blum, J.S., Marks, M.S., Cresswell, P., and Brodsky, F.M. 1990. Co-localization of molecules involved in antigen processing and presentation in an early endocytic compartment. *Nature.* 343: 133.

Guidos, C., Wong, M. and Lee, K-C. 1984. A comparison of the stimulatory activities of lymphoid dendritic cells and macrophages in T proliferative responses to various antigens. *J. Immunol.* 133: 1179.

Guillet, J.G., Lai, M.Z., Briner, T.J., Buus, S., Sette, A., Grey, H.M., Smith, J.A. and Gefter, M.L. 1987. Immunological self and non-self discrimination. *Science.* 235: 865.

Guyre, P.M., Morganelli, P.M. and Miller, P. 1983. Recombinant immune interferon increases immunoglobulin G Fc receptors cultured human mononuclear phagocytes. *J. Clin. Invest.* 72: 393.

Hackett, C.J., Dietzschold, B., Gerhard, W., Ghrist, B., Knorr, R., Gillessen, D. and Melchers, F. 1983. Influenza virus site recognized by a murine helper T cell specific for H1 strain. Localization to a nine amino acid sequences in the haemagglutinin molecule. *J. Exp. Med.* 158: 294.

Hall, J.G. and Morris, B. 1965. The origin of the cells in the efferent lymph from a single lymph node. *J. Exp. Med.* 121: 901.

Halstead, S.B. 1979. *In vivo* enhancement of Dengue virus infection in rhesus monkeys by passively transferred antibody. *J. Infect. Dis.* 140: 527.

Halstead, S.B. 1989. Antibody, macrophages dengue virus infection, shock and haemorrhage: A pathogenic cascade. *Rev. Infect. Dis.* 11: S 830.

Halstead, S.B. and O'Rourke, E.J. 1977. Dengue viruses and mononuclear phagocytes I. Infection enhancement by non-neutralizing antibodies. *J. Exp. Med.* 146: 201.

Halstead, S.B., Nimmannitya, S. and Cohen, S.N. 1970. Observations related to the pathogenesis of dengue haemorrhagic fever. IV. Relation of disease severity to antibody response and virus recovered. *Yale J. Biol. Med.* 42: 311.

Halstead, S.B., Porterfield, J.S. and O'Rourke, E.J. 1980. Enhancement of dengue virus infection in monocytes by flavivirus antisera. *Am. J. Trop. Med. Hyg.* 29: 638.

Halstead, S.B., Shotwell, H. and Casals, J. 1973. Studies on the pathogenesis of dengue infection in monkeys. II. Clinical laboratory responses to heterologous infection. *J. Infect. Dis.* 128: 15.

Harding, C.V., Cobian, F.L. and Unanue, E. 1989. Mechanisms of antigen processing. *Immunol. Rev.* 106: 77.

Harding, C.V. and Unanue, E.R. 1988. Processing and intracellular Ia: Possible role of endocytosis and protein synthesis in Ia function. *J. Immunol.* 142: 12.

Hedrick, S.M., Cohen, D.I., Nielsen, E.A. and Davis, M.M. 1984a. Isolation of cDNA clones encoding T cell specific membrane associated proteins. *Nature* 308: 149.

Hedrick, S.M., Nielsen, E.A., Kavaler, J., Cohn, D.J. and Davis, M.M. 1984b. Sequence relationships between putative T cell receptor polypeptides and immunoglobulin *Nature* 308: 153.

Henderson, B.E., Cheshire, P.P., Kirya, G.B. and Lule, M. 1970. Immunological studies with yellow fever and selected African group B arboviruses in rhesus and vervet monkeys. *Am. J. Trop. Med. Hyg.* 19: 110.

Herrmann, P., Schreier, M.H., Bazin, H., Zinkernagel, R.M. and Cerny, A. 1988. Delayed type hypersensitivity (DTH) in anti-IgM-treated B cell-depleted mice: Analysis of induction and effector phase. *Immunobiol.* 177: 382.

Hill, A. 1990. "Analysis of the cytotoxic T cell responses to flaviviruses using Kunjin-vaccinia virus recombinants". Ph.D. Thesis submitted to the Australian National University.

Hireschberg, H., Braathen, L.R. and Thorsby, E. 1982. Antigen presentation by vascular endothelium and epidermal Langerhans cells. The role of HLA-DR. *Immunol. Rev.* 66: 57.

Holt, G.D., Swiedler, S.J., Freed, J.H., and Hart, G.W. 1985. Murine Ia-associated invariant chain's processing to complex oligosaccharide forms and its dissociation from the IA^k complex. *J. Immunol.* 135: 399.

Homchampa, P., Sarasombath, S., Suvatte, V. and Vongskul, M. 1988. Natural killer cells in dengue haemorrhagic fever/dengue shock syndrome. *Asian Pacific Journal of Allergy and Immunology.* 6: 95.

Hood, L., Steinmetz, M. and Malissen, B. 1983. Genes of the major histocompatibility complex of the mouse. *Ann. Rev. Immunol.* 1: 529.

Howard, F.D. Ledbetter, J.A., Wong, J., Bieber, C.P., Stinson, E.B., Herzenberg, L.A. 1981. A human T-lymphocyte differentiation marker defined by monoclonal antibodies that block E-rosette formation. *J. Immunol.* 126: 2117.

Howard, J.C. 1985. Immunological help at last. *Nature* 314: 494.

Huang, C.H. 1982. Studies of Japanese encephalitis in China. *Adv. Virus Res.* 27: 71.

Hudson, B.W., Wolff, K. and DeMartini, J.C. 1979. Delayed-type hypersensitivity responses in mice infected with St. Louis encephalitis virus; kinetics of the response and effects of immunoregulatory agents. *Infect. Immun.* 24: 71.

Hume, D.A., Robinson, A.P., MacPherson, G.G. and Gordon, S. 1983. The mononuclear phagocyte system of the mouse defined by immunohistochemical localization of antigen F4/80. The relationship between macrophages, Langerhans cells, reticular cells and dendritic cells in lymphoid and haemopoietic organs. *J. Exp. Med.* 158: 1522.

Humes, J.L., Bonney, R.J., Pelus, L., Dahlgren, M.E., Sadowski, S.J., Kuehl, F.A.Jr. and Davies, P. 1977. Macrophages synthesize and release prostaglandins in response to inflammatory stimuli. *Nature* 269: 149.

Hurwitz, J.L., Hackette, C.J., McAndrew, E.C. and Gerhard, W. 1985. Murine Th response to influenza virus: recognition of haemagglutinin, neuraminidase, matrix and nucleoproteins. *J. Immunol.* 134: 1994.

Inaba, K. and Steinman, R.M. 1985. Protein-specific helper T lymphocyte formation initiated by dendritic cells. *Science* 229: 475.

Inaba, K. and Steinman, R.M. 1986. Accessory cell- T lymphocyte interactions. Antigen-dependent and independent clustering. *J. Exp. Med.* 163: 247.

Inaba, K., Steinman, R.M., Van Voorhis, W.C. and Muramatsus. 1983. Dendritic cells are critical accessory cells for thymus dependent antibody responses in mouse and man. *Proc. Natl. Acad. Sci. USA.* 80: 6041.

Janeway, C.A. 1988. Frontiers of the immune system. *Nature* 333: 804.

Janeway, C.A. Jr., Carding, S., Jones, B., Murray, J., Portoles, P., Rasmussen, R.R., Rojo, J., Saizawa, K., West, J. and Bottomly K. 1988. $CD4^+$ T cells: Specificity and function. *Immunol. Rev.* 101: 39.

Jenkins, M.K. and Schwartz, R.H. 1987. Antigen presentation by chemically modified splenocytes induces antigen-specific T cell unresponsiveness *in vitro* and *in vivo*. *J. Exp. Med.* 165: 302.

Jensen, P.E. 1988. Protein synthesis in antigen processing. *J. Immunol.* 141: 2545.

Jones, P., Murphy, D. and McDevitt, H.O. 1981. Variable synthesis and expression of Eα and Ae (E_β) Ia polypeptide chains in mice of different H-2 haplotypes. *Immunogenet.* 12: 321.

Jones, P.P., Murphy, D.B., Hewgill, D. and McDevitt, H.O., 1978. Detection of a common polypeptide chain in I-A and I-E sub region immunoprecipitates. *Immunochemistry* 16: 51.

Kakiuchi, T., Chesnut, R.W. and Grey, H.M. 1983. B cells as antigen presenting cells: The requirement for B cell activation. *J. Immunol.* 131: 109.

Kaplan, D.R., Griffith, R., Braciale, V.L. and Braciale, T.J. 1984. Influenza virus-specific human cytotoxic T cell clones: heterogeneity in antigen specificity and restriction by class II MHC products. *Cell Immunol.* 88: 193.

Kappler, J., Kubo, R., Haskins, K., White, J. and Marrack, P. 1983. The mouse T cell receptor: comparison of MHC restricted receptors on two T cell hybridomas. *Cell* 34: 727.

Kappler, J.W., Roehm, N. and Marrack, P. 1987 b. T cell tolerance by clonal elimination in the thymus. *Cell* 49: 273.

Kappler, J.W., Skidmore, B., White J. and Marrack, P. 1981. Antigen-inducible H-2 restricted interleukin-2 producing T cell hybridomas *J. Exp Med.* 153: 1198

Kappler, J.W., Wade, T., White, J., Kushnir, E., Blackman, M., Bill, J., Roehm, N. and Marrack, P. 1987 a. A T cell receptor V_β segment that imparts reactivity to a class II major histocompatibility complex product. *Cell* 49: 263.

Kapsenberg, M.L., Teunissen, M.B.M., Stiekema, F.E.M. and Keizer, H.G. 1986. Antigen-presenting cell function of dendritic cells and macrophages in proliferative T cell responses to soluble and particulate antigens. *Eur. J. Immunol.* 16: 345.

Katz, E.H., Hansburg, D. and Schwartz, R.H. 1983. The Ia molecules on APC plays a critical role in immune response gene regulation of T cell activation. *J. Mol. Cell. Immunol.* 1: 3.

Katz, D.H., Graves, M., Dorf, M.E., Dimuzio, H. and. Benacerraf, B. 1975. Cell interactions between histoincompatible T and B lymphocytes VII. Cooperative responses between lymphocytes are controlled by genes in the I region of the H-2 complex. *J. Exp. Med.* 141: 263.

Kaufman, J.F., Auffray, C., Korman, A.J., Shackleford, D.A.and Strominger, J. 1983. The Class II molecules of the human and murine major histocompatibility complex. *Cell* 36: 1.

Kayser, M., Klein, H., Paasch, I., Pilarski, J., Blenck, H. and Heeg, K. 1985. Human antibody response to immunization with 17 D yellow fever and inactivated TBE vaccine. *J. Med. Virol.* 17: 35.

Kelso, A. 1989. Cytokines: Structure, function and synthesis. *Current Opinion in Immunology.* 2: 215.

Kesson, A.M., Blanden, R.V. an Mullbacher, A. 1988. The secondary *in vitro*. cytotoxic T cell response to the flavivirus, West Nile. *Immunol. Cell Biol.* 66: 23.

Kesson, A.M., Blanden, R.V. and Mullbacher, A. 1987. The primary *In vivo* cytotoxic T cell response to the flavivirus, West Nile. *J. Gen. Virol.* 68: 2001.

King, N.C.J., Maxwell, L.E. and Kesson, A.M. 1989. Induction of class I major histocompatibility complex antigen expression by West Nile virus on γ interferon-refractory early murine trophoblast cells. *Proc. Natl. Acad. Sci. USA.* 86: 911.

Kisielow, P., Bluthmann, H., Stearz, U.D., Steinmetz, M. and Von Boehmer, H. 1988 a. Tolerance in T cell-receptor transgenic mice involves deletion of nonmature $CD4^{+}8^{+}$ thymocytes. *Nature* 333: 742.

Kisielow, P., Teh, H.S., Bluthmann, H., and Von Boehmer, H. 1988 b. Positive selection of antigen-specific T cells in thymus by restricting MHC molecules. *Nature* 335: 730.

Klein, J. 1975. *Biology of the mouse histocompatibility-2 complex. Springer Verlag, New York.*

Koide, S.L. and Steinman, R.M. 1987. Induction of murine interleukin 1 stimuli and responsive primary cells. *Proc. Natl. Acad. Sci. USA.* 84: 3802.

Kontny, U., Kurane, I. and Ennis, F.A. 1988. Gamma interferon augments Fcγ receptor-mediated dengue virus infection of human monocytic cells. *J. Virol.* 62: 3928.

Kourilsky, P. and Claverie, J.M. 1989. MHC-antigen interaction: What does the T cell receptor see? *Adv. Immunol.* 45: 107.

Kovac, Z. and Schwartz, R.H. 1985. The molecular basis of the requirements for antigen processing of pigeon cytochrome c prior to T cell activation. *J. Immunol.* 134: 3233.

Krensky, A.M., Robbins, E., Springer, T.A., Burakoff, S.J. 1984. LFA-1, LFA-2, and LFA-3 antigens are involved in CTL-target conjugation. *J. Immunol.* 132: 2180

Krieger, J., Jenis, D.M., Chesnut, R.W. and Grey, H.M. 1988. Studies on the capacity of intact cells and purified Ia from different B cell sources to function in antigen-presentation to T cells. *J. Immunol.* 140: 388.

Krieger, J.I. , Grammer, S.F., Grey, H.M. and Chesnut, R.W. 1985. Antigen-presentation by splenic B Cells: Resting B cells are ineffective, whereas activated B cells are effective accessory cells for T cell responses. *J. Immunol.* 135: 2937.

Krieger, J.I., Chesnut, R.W. and Grey, H.M. 1986. Capacity of B cells to function as stimulators of a primary mixed leukocyte reaction. *J. Immunol.* 137: 3117.

Kupfer, A. and Singer, S.J. 1988. Molecular dynamics in the membranes of helper T cells. *Proc. Natl. Acad. Sci. USA.* 85: 8216.

Kupfer, A., Singer, S.J., Janeway, C.A.Jr. and Swain, S.L. 1987. Co-clustering of CD4 (L3T4) molecule with the T-cell receptor is induced by specific direct interaction of helper T cells and antigen-presenting cells. *Proc. Natl. Acad. Sci. USA*. 84: 5888.

Kurane, I., Hebblewaite, D., Brandt, W. and Ennis, F.A. 1984. Lysis of dengue virus-infected cells by natural cell-mediated cytoxocity and antibody-dependent cell-mediated cytotoxicity. *J. Virol.* 52: 223.

Kurane, I., Innis, B.L., Nisalak, A., Hoke, C., Nimmannitya, S., Meager, A. and Ennis, F.A. 1989. Human T cell responses to dengue virus antigens. *J. Clin. Invest.* 83: 506.

Lamb, J.R., Skidmore, B.J., Green, N., Chiller, J.M. and Feldmann, M. 1983. Induction of tolerance in influenza virus-immune T lymphocyte clones with synthetic peptides of influenza haemagglutinin. *J. Exp. Med.* 157: 1434.

Lanzavecchia, A. 1985. Antigen specific interaction between T and B cells. *Nature* 314: 537.

Lanzavecchia, A. 1987. Antigen uptake and accumulation in antigen-specific B cells. *Immunol. Rev.* 99: 39.

Lassila, O., Vainio, O. and Matzinger, P. 1988. Can B cells turn on virgin T cells? *Nature* 334: 253.

Lee, P., Matsueda, G.R. and Allen, P.M. 1988. T cell recognition of fibrinogen : A determinant on the Aα-chain does not require processing. *J. Immunol.* 140: 1063.

Littman, D.R. 1987. The structure of the CD4 and CD8 genes. *Ann. Rev. Immunol.* 5: 561.

Liu, Y., King, N., Kesson, A., Blanden, R.V. and Mullbacher, A. 1989. Flavivirus infection upregulates the expression of class I and class II major histocompatibility antigens on and enhances T cell recognition of astrocytes *in vitro*. *J. Neuroimmunol.* 21: 157.

Livingstone, A.M. and Fathman, C.G. 1987. The structure of T-cell epitopes. *Ann. Rev. Immunol.* 5: 477.

Long, E. 1989. Intracellular traffick and antigen processing. *Immunol. Today*. 10: 232.

Lorenz, R.G. and Allen, P.M. 1988. Processing and presentation of self proteins. *Immunol. Rev*. 106: 115.

Lorenz, R.G., Blum, J.S. and Allen, P.M. 1990. Constitutive competition by self proteins from antigen presentation can be overcome by receptor-enhanced uptake. *J. Immunol.* 144: 1600.

Macatonia, S.E., Taylor, P.M., Knight, S.C. and Askonas, B.A. 1989. Primary stimulation by dendritic cells induces anti-viral proliferative and cytotoxic T cell responses *in vitro*. *J. Exp. Med*. 169: 1255.

MacDonald, H.R., Hengartner, and Pedrazzini, T. 1988. Intrathymic deletion of self-reactive cells prevented by neonatal anti-CD4 antibody treatment. *Nature* 335: 174.

Macfarlan, R.I. and White, D.O. 1980. Further characterization of natural killer cells induced by Kunjin virus. *Aust. J. Exp. Biol. Med. Sci*. 58: 77.

Mackow, E., Makino, Y., Zhao, B., Zhang, Y-M., Markoff, L., Buckler-White, A., Guiler, M., Chanock, R. and Lai, C-J. 1987. The nucleotide sequence of dengue type 4 virus: analysis of genes coding for nonstructural proteins. *Virology* 159: 217.

Madrid, DE, A.T. and Porterfield, J.S. 1974. The flaviviruses (group B arboviruses): a cross-neutralization study. *J. Gen. Virol*. 23: 91.

Mage, M.G. 1980. Preparation of Fab fragments from IgGs of different animal species. *Methods in Enzymology*. 70: 142.

Maizels, R.M., Clarke, J.A., Harvey, M.A., and Sercarz, E.E. 1980. Epitope specificity of T cell proliferative response to lysozyme. Proliferative T cells react predominantly to different determinants from those recognized by B cells. *Eur. J. Immunol*. 10: 509.

Manca, F., Fenoglio, D., Kunke, A., Cambiaggi, C., Sasso, M., and Celada, F. 1988. Differential activation of T cell clones stimulated by macrophages exposed to antigen complexed with monoclonal antibodies. *J. Immunol.* 140: 2893.

Manca, F., Kunkl, A., Fenoglio, D., Fowler, A., Sercaz, E., Celada, F. 1985. Constraints in T-B cooperation related to epitope topology in E.coli. β galactosidase. I The fine specificity of T cells dictates the fine specificity of antibody directed to conformation-dependent determinants. *Eur. J. Immunol.* 15: 345.

Marrack, P., Kushnir, E., Born, W., McDuffie, M., and Kappler, J. 1988.a The development of helper T cell precursors in mouse thymus. *J. Immunol.* 140: 2508.

Marrack, P., Lo, D., Brinster, R., Palmiter, R., Burkly, L., Flavell, R.H. and Kappler, J. 1988.b The effect of thymus environment on T cell development and tolerance. *Cell* 53: 627.

Marshall, I.D., Woodroofe, G.M. and Hirsch, S. 1982. Viruses recovered from mosquitoes and wildlife serum collected in the Murray Valley of South-eastern Australia, February 1974, during an epidemic of encephalitis. *Aust. J. Exp. Biol. Med. Sci.* 60: 457.

Marusic- Galesic, S., Longo, D.L. and Kruisbeek, A.M. 1989. Preferred differentiation of T-cell receptor specificities based on the MHC glycoproteins encountered during development: evidence for positive selection. *J. Exp. Med.* 169: 1840.

Mathur, A., Arora, K.L. and Chaturvedi, U.C. 1983. Host defence mechanisms against Japanese encephalitis virus infection in mice. *J. Gen. Virol.* 64: 805.

Matis, L.A., Glimcher, L.H., Paul, W.E. and Schwartz, R.H. 1983. Magnitude of response of histocompatibility-restricted T-cell clones is a function of the product of the concentrations of antigen and Ia molecules. *Proc. Natl. Acad. Sci. USA.* 80: 6019.

McDevitt, H.O., Deak, B.D., Shreffler, C., Klein., J., Sumpfling, H., and Snell, G.D. 1972. Genetic control of immune response. *J. Exp. Med.* 135: 1259.

McIntyre, B.W. and Allison, J.P. 1983. The mouse T cell receptor: structural heterogeneity of molecules on normal T cells defined by xenoantiserum. *Cell* 24: 739..

Meuer, S.C., Fitzgerald, K.A., Hussey, P.E., Hodgan, J.C., Scholssman, S.F., and Reinberz, E.L. 1983. Clonotypic structures involved in antigen specific human T cell function. Relationship to the T_3 molecular complex. *J. Exp. Med.* 157: 705.

Miller, J.F.A.P., Vadas, M.A., Whitelaw, A., and Gamble, J. 1975. H-2 gene complex restricts transfer of delayed type hypersensitivity in mice. *Proc. Natl. Acad. Sci. USA.* 72: 5095.

Mills, G., Monticone, V. and Paetkau, V. 1976. The role of macrophages in thymocyte mitogenesis. *J. Immunol.* 117: 1325.

Mims, C.A. and Blanden, R.V. 1972. Antiviral action of imune lymphocytes in mice infected with lymphocytic choriomeningitis virus. *Infect. Immun.* 6: 695.

Miyazaki, H. and Osawa, T. 1983. Accessory functions and mutual cooperation of murine macrophages and dendritic cells. *Eur. J. Immunol.* 13: 984.

Mizel, S.B. 1981. Production and quantitation of lymphocyte-activating factor (interleukin-1). In *Manual of Macrophage Methodology. Collection, characterization and function. Ed. Herscowitz, H.B., Holden, H.T., Bellanti, J.A., and Ghaffar, A. Marcel Dekker, Inc; New York and Basal,* pp. 407.

Monath, T.P. 1986. Pathology of flaviviruses. In: *The Togaviridae and Flaviviridae. Ed. Schlesinger, S. and Schlesinger, M.J., Plenum Press, New York,* pp 375.

Monath, T.P. 1985. Flaviviruses. In *"Virology", Fields, B.N. (Ed)., Ravan Press, New York,* pp. 955.

Monroe, J.G. and Cambier, J.C. 1983. Level of mIa expression on mitogen-stimulated murine B lymphocytes is dependent on position in cell cycle. *J. Immunol.* 130: 626.

Monroe, J.G., Lowy, A., Granstein, R.D. and Greene, M.I. 1984. Studies of immune responsiveness and unresponsiveness to the *p*-azobenzenearsonate (ABA) hapten. *Immunol. Rev.* 80: 103.

Moore, M.W., Carbone, F.R. and Bevan, M.J. 1988. Introduction of soluble protein into class I pathway of antigen processing and presentation. *Cell* 54: 777.

Morrison, L.A., Braciale, V.L., and Braciale, T.J. 1988. Antigen form influences induction and frequency of influenza-specific class I and class II MHC-restricted cytolytic T lymphocytes. *J. Immunol.* 141: 363.

Morrison, L.A., Lukacher, A.E., Braciale, V.L., Fan, D.P. and Braciale, T.J. 1986. Differences in antigen presentation to MHC Class-I and Class II-restricted influenza virus-specific cytolytic T lymphocytic clones. *J. Exp. Med.* 163: 903.

Mosier, D.E. 1967. A requirement for two cell types for antibody formation *in vitro*. *Science* 158: 1573.

Mosmann, T.R. and Coffman, R.L. 1989. Heterogeneity of cytokine secretion pattern and function of helper T cells. *Adv. Immunol.* 46: 111.

Mosmann, T., Cherwinski, H., Bond, M.W., Giedlin, M. and Coffman, R.L. 1986. Two types of murine T helper cell clones. I. Defining according to profile of lymphokine activities. *J. Immunol.* 136: 2348.

Mullbacher, A., Blanden, R.V. and Brenan, M. 1983. Neonatal tolerance of major histocompatibility complex antigens alters Ir gene control of the cytotoxic T cell response to vaccinia virus. *J. Exp. Med.* 157: 1324.

Muller, D., McDonald, M., Stallman, N. and King, J. 1986. Kunjin virus encephalomyelitis. *Med. J. Aust.* 144: 41.

Nabholz, M., Young, H., Rynbeek, A., Boccardo, R., David, C.S., Meo, T., Miggiano, V. and Shreffler, D.C. 1975. I-region associated determinants: expression on mitogen stimulated lymphocytes and detection by cytotoxic T-cells. *Eur. J. Immunol.* 5: 594.

Nedwin, G.E., Svedersky, L.P., Bringman, T.S., Palladino, M.A.Jr. and Goeddel, D.V. 1985. Effect of interleukin-2, interferon-γ and mitogens on the production of TNF α and β. *J. Immunol.* 135: 2492.

Nussenzweig, M.C. and Steinman, R.M. 1980. Contribution of dendritic cells to stimulation of the murine syngeneic mixed leukocyte reaction. *J. Exp. Med.* 151: 1196.

Nussenzweig, M.C., Steinman, R.M., Unkeless, J.C., Witmer, M.D., Gutchinov, B. and Cohn, Z.A. 1981. Studies on the cell surface of mouse dendritic cells and other leukocytes. *J. Exp. Med.* 154: 168.

Nussenzweig, M.C., Steinman, R.M., Witmer, M.D. and Gutchinov, B. 1982. A monoclonal antibody specific for mouse dendritic cells. *Proc. Natl. Acad. Sci. USA.* 79: 161.

O'Garra, A., Umland, S., De France, T. and Christiansen, J. !988. 'B cell factors' are pleiotropic. *Immunology Today.* 9: 45.

Okada, M., Kitahara, M., Kishimoto, S., Matsuda, T. and Kishimoto, T. 1988. IL-6/BSF-2 functions as a killer helper factor in the in-vitro induction cytotoxic T lymphocytes. *J. Immunol.* 141: 1543.

Oldstone, M.B.A., Belount, P., Southern, P.J. and Lampert, P.W. 1986. Cytoimmunotherapy for persistent virus infection reveals a unique clearance pattern from the central nervous system. *Nature* 321: 239.

Ozato, K., Evans, G.A., Shykind, B., Marguiles, D.H. and Seidman, J.C. 1983. Hybrid H-2 histocompatibility gene products assign domains recognized by alloreactive T cells. *Proc. Natl. Acad. Sci. USA.* 80: 2040.

Pang, T., Devi, S., Blanden, R.V. and Lam, S.K. 1988. T Cell-mediated Cytotoxicity against Dengue-infected target cells. *Microbiol. Immunol.* 32: 511.

Pang, T., Wong, P.Y. and Pathmanathan, R. 1982. Induction and characterization of delayed-type hypersensitivity to Dengue virus in mice. *J. Infect. Dis.* 146: 235.

Parish, C.R. and McKenzie, I.F.C. 1978. A sensitive rosetting method for obtaining subpopulations of lymphocytes which react with alloantisera. *J. Immunol. Methods.* 20: 173.

Peiris, J.S.M. and Porterfield, J.S. 1979. Antibody-mediated enhancement of flavivirus replication in macrophage cell lines. *Nature* 282: 509.

Pereira, R.A. 1986. Studies on lymphoid dendritic cells. Ph.D. Thesis submitted to the Australian National University.

Pereira, R.A., King, N.C.J. and Blanden, R.V. 1986. Comparison of functional properties of thymic and splenic dendritic cells. *Cell. Immunol.* 102: 152.

Phillpotts, R.J. and Porterfield, J.S. 1985. Antibody dependent enhancement of tick-borne encephalitis virus infectivity. *J. Gen. Virol.* 66: 831.

Pincus, M.R., Gerewitz, F., Schwartz, R.H., Scheraga, H.A. 1983. Correlation between the conformation of cytochrome c peptides and their stimulatory activity in a T lymphocyte proliferation assay. *Proc. Natl. Acad. Sci. USA.* 80: 3297.

Poo, W.J., Conrad, L. and Janeway, C.A. 1988. Receptor-directed focusing of lymphokine release by helper T cells. *Nature* 332: 378.

Price, W.H., Parks, J., Ganaway, J., Lee, R. and O'Leary, W. 1963. A sequential immunization procedure against certain group B arboviruses. *Am. J. Trop. Med. Hyg.* 12: 624.

Rabin, E.M., Mond, J.J., Ohara, J. and Paul, W.E. 1986. Interferon γ inhibits the action of B cell stimulatory factor (BSF)-1 on resting B cells. *J. Immunol.* 137: 1573.

Ragoff, T. and Lipsky, P.E. 1979. Characterization of isolated guinea pig Kupffer cells: accessory function in mitogen-induced T lymphocyte activation. *J. Immunol.* 123: 1920.

Ramila, G., Sklenar, I., Kennedy, M., Sunshine, G.H. and Erb, P. 1985. Evaluation of accessory cell heterogeniety. II. Failure of dendritic cells to activate antigen-specific T helper cells to soluble antigens. *Eur. J. Immunol.* 15: 189.

Rammemsee, H.G., Kroschewski, R. and Frangoulis, B. 1989. Clonal anergy induced in mature Vβ6$^+$ T lymphocytes on immunizing *Mls-1^b* mice with Mls-1^a expressing cells. *Nature* 339: 541.

Raulet, D.H. 1989. Antigens for γδ T cells. *Nature* 339: 342.

Reinherz, E.L., Kung, P., Goldstein, G. and Schlossman, S. 1979. Separation of functional subsets of T cells by monoclonal antibody. *Proc. Natl. Acad. Sci. USA.* 76: 4061.

Rice, C.M. 1986. Structure of flavivirus genome. In *"The Togaviridae and Flaviviridae". Ed. Schlesinger, S. and Schlesinger M.J. Plenum Press, New York, London,* pp.379..

Rice, C.M., Lenches, E.M., Eddy, S.R., Shin, S.J., Sheets, R.L. and Strauss, J.H. 1985. Nucleotide sequence of yellow fever virus: implication for flavivirus gene expression and evolution. *Science.* 229: 726.

Robertson, M. 1982. The evolutionary past of the major histocompatibility complex and the future of cellular immunology. *Nature* 297: 629.

Roehrig, J.T. 1986. The use of monoclonal antibodies in studies of the structural proteins of togaviruses and flaviviruses. In *"The Togaviridae and Flaviviridae". Ed. Schlesinger, S. and Schlesinger M.J. Plenum Press, New York, London,* pp.251.

Roehrig, J.T., Mathew, J.H., and Trent, D.W. 1983. Identification of epitopes on the E glycoprotein of St. Louis encephalitis virus using monoclonal antibodies. *Virology* 128: 118.

Ron, Y. and Sprent, J. 1987. T cell priming *In vivo*: a major role for B cells in presenting antigen to T cells in lymph nodes. *J. Immunol.* 138: 2848.

Ron, Y., De Batslinger, P., Gordon, J., Feldman, M. and Segal, S. 1981. Defective induction of antigen reactive T cells in B cell-deprived mice. *Eur. J. Immunol.* 11: 964.

Romani, N., Koide, S., Crowley, M.W., Livingstone, A.M., Fathman, C.G., Inaba, K. and Steinman, R.M. 1989. Presentation of exogenous protein antigens by dendritic cells to T cell clones. Intact protein is presented best by immature, epidermal Langerhans cells. *J. Exp. Med.* 169: 1169.

Rosenthal, A.S., Barcinski, M.A., and Blake, J.T. 1977. Determinant selection is a macrophage dependent immune response gene function. *Nature* 267: 156.

Rothbard, J.B. and Taylor, W.R. 1988. A sequence pattern common to T cell epitopes. *EMBO*. 7: 93.

Rothman, A., Kurane, I., Zang, Y., Lai, C.J. and Ennis, F.A. 1989. Dengue virus-specific murine T-lymphocyte proliferation: Serotype specificity and response to recombinant viral proteins. *J. Virol.* 63: 2486.

Saito, H., Kranz, D.M., Takagaki, Y., Hayday, A.C., Eisen, H.N. and Tonegawa, S. 1984. Complete structure of a heterodimeric T-cell receptor deduced from cDNA sequences. *Nature* 309: 757.

Samelson, L.E., Harford, T.B. and Klausner, R.D. 1985. Identification of the components of the murine T cell receptor complex. *Cell* 43: 223.

Schlesinger, J.J., Brandriss, M.W. and Walsh, E.E. 1985. Protection against 17D yellow fever encephalitis in mice by passive transfer of monoclonal antibodies to the nonstructural glycoprotein gp 48 and by active immunization with gp 48. *J. Immunol.* 135: 2805.

Schlesinger, J.J., Brandriss, M.W., Cropp, B.C. and Monath, T.P. 1986. Protection against yellow fever in monkeys by immunization with yellow fever virus non-structural protein NSI. *J. Virol.* 60: 1153.

Schwartz, R. 1986. Immune response (Ir) genes of the murine major histocompatibility complex. *Adv. Immunol.* 38: 31.

Schwartz, R.H. 1978. A clonal deletion model for Ir gene control of the immune response. *Scand. J. Immunol.* 7: 3.

Schwartz, R.H., Fox, B.S., Fraga, E., Chen, C., Singh, B. 1985. The T lymphocyte response to cytochrome c. V. Determination of the minimal peptide size required for stimulation of T cell clones and assessment of the contribution of each residue beyond this size to antigenic potency. *J. Immunol.* 135: 2598.

Segal, D.M., Titus, J.A. and Dower, S.K. 1983. The FcR-mediated endocytosis of model immune complexes by T cells from the P388D1 mouse macrophage line. II. The role of ligand-induced self-aggregation in promoting internalization. *J. Immunol.* 130: 138

Sercarz, E.E., Yowell, R.L., Turkin, D., Miller, a., Araneo, B.A. and Adorini, L. 1978. Different functional specificity repertoires for suppressor and helper T cells. *Immunol. Rev.* 39: 108.

Shastri, N., Miller, A. and Sercarz, E.E. 1986. Amino acid residues distinct from the determinant region can profoundly affect activation of T cell clones by related antigens. *J. Immunol.* 136: 371.

Shaw, S., Luce, G.E.G., Quinones, R., Gress, R.E., Springer, T.A., Sanders, M.E. 1986. Two antigen-independent adhesion pathways used by human cytotoxic T cell clones. *Nature* 323: 262.

Sheets, P., Schwartz, A., Jacoby, R.O. and Bhatt, P.N. 1979. T cell-mediated cytotoxicity for L-929 fibroblasts infected with Banzi virus (flavivirus). *J. Infect. Dis.* 140: 384.

Shreffler, D.C., David, C.S., Cullen, S.E., Frelinger, J.A. and Neiderhuber, J.E. 1976. Serological and functional evidence for further subdivisions of the I-regions of the H-2 complex. *Cold Spring Harbour Symp. Quant. Biol.* 41: 477.

Smith, G.W. and Wright, P.J. 1985. Synthesis of proteins and glycoproteins in dengue type 2 virus-infected Vero and Aedes albopictus cells. *J. Gen. Virol.* 66: 559.

Snapper, C.M. and Paul, W.E. 1987.a Interferon γ and B cell stimulatory factor-1 reciprocally regulate Ig isotype production. *Science* 236: 944.

Snapper, C.M. and Paul, W.E. 1987 b. B cell stimulatory factor-1 (interleukin 4) prepares resting murine B cells to secrete IgG1 upon subsequent stimulation with bacterial polysaccharide. *J. Immunol.* 139: 10.

Snell, G.D., Dausset, J. and Nathenson, S. 1976. *Histocompatibility. Academic Press. London.* 1.

Snider, D.P. and Segal, D.M. 1987. Targeted antigen presentation using crosslinked antibody heteroaggregates. *J. Immunol.* 139: 1609.

Solinger, A.M., Ultee, M.E., Margoliash, E. and Schwartz, R.H. 1979. T lymphocyte response to cytochrome c. I. Demonstration of a T-cell heteroclitic proliferative response and identification of a topographic antigenic determinants on pigeon cytochrome c whose immune recognition requires two complementing major histocompatibility complex-linked immune response genes. *J. Exp. Med.* 150: 830.

Sprent, J. 1973. Circulating B and T lymphocytes of the mouse. I migratory properties. *Cell. Immunol.* 7: 10.

Sprent, J., Lo, D., Gao, E-K. and Ron, Y. 1988. T cell selection in the thymus. *Immunol. Rev.* 101: 173.

Steinman, R.M. and Cohn, Z.A. 1973. Identification of a novel cell type in peripheral lymphoid organs of mice. I morphology, quantitation, tissue-distributions. *J. Exp. Med.* 137: 1142.

Steinman, R.M. and Witmer, M. 1978. Lymphoid dendritic cells are potent-stimulators of primary mixed leukocyte reaction in mice. *Proc. Natl. Acad. Sci. USA.* 75: 5132.

Steinman, R.M., Adams, J.C. and Cohn, Z.A. 1975. Identification of a novel cell type in peripheral lymphoid organs of mice. IV Identification and distribution in mouse spleen. *J. Exp. Med.* 141: 804.

Steinman, R.M., Gutchinov, B., Witmer, M.D., Nussenzweig, M.C. 1983. Dendritic cells the principal stimulators of the mixed leukocyte reaction in mice. *J. Exp. Med.* 157: 613.

Steinman, R.M., Kaplan, G., Witmer, M.D. and Cohn, Z.A. 1979. Identification of a novel cell type in peripheral lymphoid organs of mice V Purification of splenic dendritic cells, new surface markers, and maintenance *in vitro*. *J. Exp. Med.* 149: 1.

Steinman, R.M., Lustig, D.S. and Cohn, Z.A. 1974. Identification of a novel cell type in peripheral lymphoid organs of mice. III Functional properties *in vivo J. Exp. Med.* 139: 1431.

Steinmetz, M., Winoto, A., Minrad, K. and Hood, L. 1982. Clusters of genes encoding mouse transplantation antigens. *Cell* 28: 489.

Stingl, G., Katz, S.I., Clement, L., Green, I. and Shevach, E.M. 1978. Immunologic function of Ia-bearing epidermal Langerhans cells. *J. Immunol.* 121: 2005.

Stockinger, B., Pessara, U., Lin, R.H., Habicht, J., Grez, M., and Koch, N. 1989. A role of Ia-associated invariant chains in antigen processing and presentation. *Cell* 56: 683.

Streicher, H., Berkower, I.J., Busch, M., Gurd, F. and Berzofsky, J.A. 1984. Antigen conformation determines processing requirements for T-cell activation. *Proc. Natl. Acad. Sci. USA*. 81: 6831.

Strominger, J.L. 1989. The γ-δ T cell receptor and class Ib MHC-related proteins: Enigmatic molecules of immune recognition. *Cell* 57: 895.

Stroynowski, Y., Orn, A., Goodnow, R.S., McMillan, M., Forman, J., Brayton, P.R., Frelinger, J. and Hood, L. 1984. Cytotoxic T lymphocytes recognize determinants on the BALB/c H-2L^{d} molecule controlled by α_1 and α_2 but not α_3 external domains. *Immunogenetics*. 20: 141.

Sumiyoshi, H., Mori, C., Fuke, I., Morita, K., Kuhara, S., Kondou, J., Kikuchi, Y., Nagamatu, H. and Igarashi, A. 1987. Complete nucleotide sequence of the Japanese Encephalitis virus genome. *Virology*. 161: 497.

Sunshine, G.H., Gold, D.P., Wortis, H.H., Marrack, P. and Kappler, J.W. 1983. Mouse spleen dendritic cells present soluble antigens to antigen-specific T cell hybridoma. *J. Exp. Med.* 158: 1745.

Swain, S. L. 1983. T cell subsets and the recognition of MHC. *Immunol. Rev.* 74: 129.

Swain, S.L. 1981. Significance of Lyt phenotypes: Lyt2 antibodies block activities of T cells that recognize class I major histocompatibility complex antigens regardless of their function. *Proc. Natl. Acad. Sci. USA.* 78: 7101.

Swain, S., Dutton, R., McKenzie, D., Helstrom, H. and English, M. 1988. Role of antigen in B cell response: Specific antigen and the lymphokine IL-5 synergize to drive B cell lymphoma proliferation and differentiation to Ig secretion. *J. Immunol.* 140: 4224.

Tanford, C. 1968. Protein denaturation . *Adv. Protein Chemistry.* 23: 122.

Taylor, W.P. and Marshall, I.D. 1975. Adaptation studies with Ross River virus: Laboratory mice and cell cultures. *J. Gen. Virol.* 68: 59.

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Teh, H.S., Kisielow, P., Scott, B., Kishi, H., Uematsu, Y., Bluthmann, H., and Von Boehmer, H. 1988. Thymic major histocompatibility complex antigens and the α,β T-cell receptor determine the CD4/CD8 phenotype of T cells. *Nature* 335: 229.

Tew, J.G., Kosco, M.H., and Szakal, A.K. 1989. The alternative antigen pathway. *Immunology Today.* 10: 229.

Ting, J.P.Y. Shigekawa, B.L., Linthicum, D.S., Weiner, L.P. and Frelinger, J.A. 1981. Expression and synthesis of murine immune response associated (Ia) antigens by brain cells. *Proc. Natl. Acad. Sci. USA.* 78: 3170.

Tite, J.P., Foellmer, H.G., Madri, J.A. and Janeway, C.A. Jr. 1987. Inverse Ir gene control of the antibody and T cell proliferative responses to human basement membrane collagen. *J. Immunol.* 139: 2892.

Tony, H., Phillips, N.E. and Parker, D.C. 1985. Role of membrane immunoglobulin (Ig) crosslinking in membrane Ig-mediated major histocompatibility-restricted T cell-B cell cooperation. *J. Exp. Med.* 162: 1695.

Tony, H.P. and Parker, D.C. 1985. MHC-restricted polyclonal B cell responses resulting from helper T cell recognition of anti-immunoglobulin presented by small B lymphocytes. *J. Exp. Med.* 161: 223.

Townsend, A.R.M. Bastin, J., Gould, K., and Brownlee, G.G. 1986a. Cytotoxic T lymphocytes recognize influenza haemagglutinin that lacks a signal sequence. *Nature* 324: 575

Townsend, A.R.M., Rothbard, J., Gotch, F.M., Bahadur, G., Wraith, D. and McMichael, A.J. 1986b. The epitopes of influenza nucleoprotein recognized by cytotoxic T lymphocytes can be defined with short synthetic peptides. *Cell* 44: 959.

Trent, D.W., Kinnet, R.M., Johnson, B.J.B., Vorndnam, A.V., Grant, J.A., Deubel, V., Rice, C.M. and Hahn, C. 1987. Partial nucleotide sequence of St. Louis encephalitis virus RNA: structural proteins, NS1, ns2a, and ns2b. *Virology*. 156: 293.

Unanue, E. 1984. Antigen-presenting function of the macrophges. *Ann. Rev. Immunol.* 2: 395.

Unanue, E.R. 1981. The regulatory role of macrophages in antigenic stimulation. Part Two: Symbiotic relationship between lymphocytes and macrophages. *Adv. Immunol.* 31: 1.

Uren, M.F., Doherty, P.C. and Allan, J.E. 1987. Flavivirus-specific murine L3T4^{+} T cell clones: induction, characterization and cross-reactivity. *J. Gen Virol.* 68: 2655.

Vane, J.R. 1971. Inhibition of prostaglandin synthesis as mechanism of action for aspirin-like drugs. *Nature, New Biol.* 231: 232.

Verrman, A.J.P. 1974. On the interdigitating cells in the thymus dependent area of the rat spleen: a relation between the mononuclear phagocyte system and T-lymphocytes. *Cell Tissue Res.* 148: 247.

Vitetta, E.S., Botran, R.F., Myess, C.D. and Sanders, V.M. 1989. Cellular interactions in the humoral immune response. *Adv. Immunol.* 45: 1.

Von Boehmer, H. 1986. The selection of the α,β heterodimeric T-cell receptor for antigen. *Immunolology Today.* 7: 333.

Von Boehmer, H., Teh, H.S. and Kisielow, P. 1989. The thymus selects the useful, neglects the useless and destroys the harmful. *Immunology Today.* 10: 57.

Wagner, H., Gotze, D., Ptschelinzew, L. and Rollinghof, M. 1975. Induction of cytotoxic T lymphocytes against I-region coded determinants: *in vitro.* evidence for a third histocompatibility locus in the mouse. *J. Exp. Med.* 142: 1477.

Waldron, J.A. Jr., Horn, R.G. and Rosenthal, A.S. 1974. Antigen-induced proliferation of guinea pig lymphocytes *in vitro.*: Functional aspects of antigen handling by macrophages. *J. Immunol.* 112: 746.

Werdelin, O. 1986. Determinant protection. A hypothesis for the activity of immune response genes in the processing and presentation of antigens by macrophages. *Scand. J. Immunol.* 24: 625.

Werdelin, O. 1982. Chemically related antigens compete for presentation by accessory cells to T cells. *J. Immunol.* 129: 1883.

Werdelin, O., Mouritsen, S., Petersen, B.L., Sette, A. and Buss, S. 1988. Facts on the fragmentation of antigens in presenting cells and on the association of antigen fragments with MHC molecules in cell-free system and speculation on the cell biology of antigen processing. *Immunol. Rev.* 106: 181.

Westway, E.G. 1965. The neutralization of arboviruses. II Neutralization in heterologous virus-serum mixtures with four group B arboviruses. *Virology* 26: 528.

Xin, Y.Y., Ming, Z.G., Peng, G.Y., Jian, A. and Min, L.H. 1988. Safety of a live-attenuated Japanese encephalitis virus vaccine (SA_{14}-14-2) for children. *Am. J. Trop. Med. Hyg.* 39: 214.

Yamamoto, K., Koch, N., Steinmetz, M. and Hammerling, G.J. 1985. One gene encodes two distinct Ia-associated invariant chains. *J. Immunol.* 134: 3461.

Yanagi, Y., Yoshikai, Y., Leggett, K., Clark, S.P., Aleksander, I. and Mak, T.W. 1984. A human T cell specific cDNA clone encodes a protein having extensive homology to immunoglobulis chains. *Nature* 308: 145.

Yap, K.L. and Ada, G.L. 1977. Cytotoxic T cells specific for influenza virus-infected target cells. *Immunology* 32: 151.

Yewdell, J.W., Bennink, J.R. and Hoska, Y. 1988. Cells process exogenous proteins for recognition by cytotoxic T lymphocytes. *Science*. 239: 637.

Yokoyama, K. and Nathenson, S.G. 1983. Intramolecular organization of class I H-2 MHC antigens: localization of the alloantigenic determinants and the β2 m binding site to different regions of the H-$2k^b$ glycoprotein. *J. Immunol.* 130: 1419.

Yoshikawa, M., Watanbe, M. and Hozumi, N. 1987. Analysis of proteolytic processing during specific antigen presentation. *Cell Immunol.* 110: 431.

Zhang, Y., Hayes, E.P., McCarty, T.C. Dubois, D.R., Summers, P.L., Eckels, K.H., Chanock, R.M. and Lai, C.J. 1988. Immunization of mice with dengue structural protein NS_1 expressed by baculovirus recombinant induces resistance to dengue virus encephalitis. *J. Virol.* 62: 3027

Ziegler, H.K. and Unanue, E.R. 1982. Decrease in macrophage antigen catabolism caused by ammonia and chloroquine is associated with inhibition of antigen presentation to T cells. *Proc. Natl. Acad. Sci. USA*. 79: 175.

Ziegler, K. and Unanue, E.R. 1981. Identification of a macrophage antigen-processing event required for I-region-restricted antigen presentation to T lymphocytes. *J. Immunol.* 127: 1869.

Zinkernagel, R.M., Callahan, G.N., Klein, J. and Dennert, G. 1978. Cytotoxic T cells learn specificity for self H-2 during differentiation in the thymus. *Nature* 271: 251.

Zinkernagel, R.M. and Doherty, P.C. 1973. Cytotoxic thymus derived lymphocytes in CSF of mice with lymphocytic choriomeningitis. *J. Exp. Med.* 138: 1266.

Zinkernagel, R.M. and Doherty, P.C. 1974. Immunological surveillance against altered self components by sensitized T lymphocytes in lymphocytic choriomeningitis. *Nature*. 251: 547.

Zinkernagel, R.M. and Doherty, P.C. 1979. MHC-restricted cytotoxic T cells: studies on the biological role of polymorphic major transplantation antigens determining T-cell restriction specificity function and responsiveness. *Adv. Immunol.* 27: 51.